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13. ABSTRACT *(Maximum 200 words)*

The problem investigated was to test the hypothesis that neurotoxins initiate a cascade of events that converge on the redox mechanisms common to brain injury. Neurotoxins (e.g., soman, kainic acid, cyanide, etc.) initiate biochemical changes in brain that lead either to marked hyperactivity (i.e., soman- or kainic acid-induced seizures) or hypoactivity (i.e., cyanide-induced comatose) of brain regions. In both situations, protective mechanisms are activated to conserve energy, but eventually excitotoxic driven events ensue leading to an influx of calcium (i.e., calcium stress) and water movements (i.e., osmotic stress). These stresses converge on the brain redox systems. **Task 1** dealt with detection of biomarkers for free radicals in cerebral extracellular fluid via microdialysis and in regional brain tissues. Both cyanide and soman cause marked changes in ascorbate and urate. Kainic acid-induced seizures increase nitric oxide formation. Soman increased "catalytic iron" and decreased tissue glutathione. **Task 2** dealt with detection of tissue biomarkers of free radical responses by gene expression studies. Kainic acid, a surrogate seizuregenic compound, changes metallothionein-1, heme oxygenase-1, c-fos, heat shock protein-70 and interleukin-1 gene expression in brain. Soman caused marked changes in metallothionein-1 and heme oxygenase-1. Clearly, the redox state is important in neurotoxin-induced brain damage.

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Soman, cyanide, kainic acid, ascorbate, urate, glutathione, monoamines, iron, redox chemistry, nitric oxide, gene expression, metallothionein, heme oxygenase

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INTRODUCTION

Neurotoxins (e.g., soman, kainic acid, cyanide, etc.) initiate biochemical changes in brain that lead either to marked hyperactivity (i.e., soman- or kainic acid-induced seizures) or hypoactivity (i.e., cyanide-induced comatose) of brain regions. In both situations, protective mechanisms are activated to conserve energy, but eventually excitotoxic driven events ensue leading to an influx of calcium (i.e., calcium stress) and water movements (i.e., osmotic stress). These stresses converge on the brain redox systems. Perturbation of redox systems can lead to intermediates that can damage critical targets (e.g., lipids, proteins, DNA, etc.) via free radicals, a final common pathway for many types of brain injury (Reviewed in Pazdernik et al., 1992, 1994). Our working hypothesis is illustrated in Figure 1.

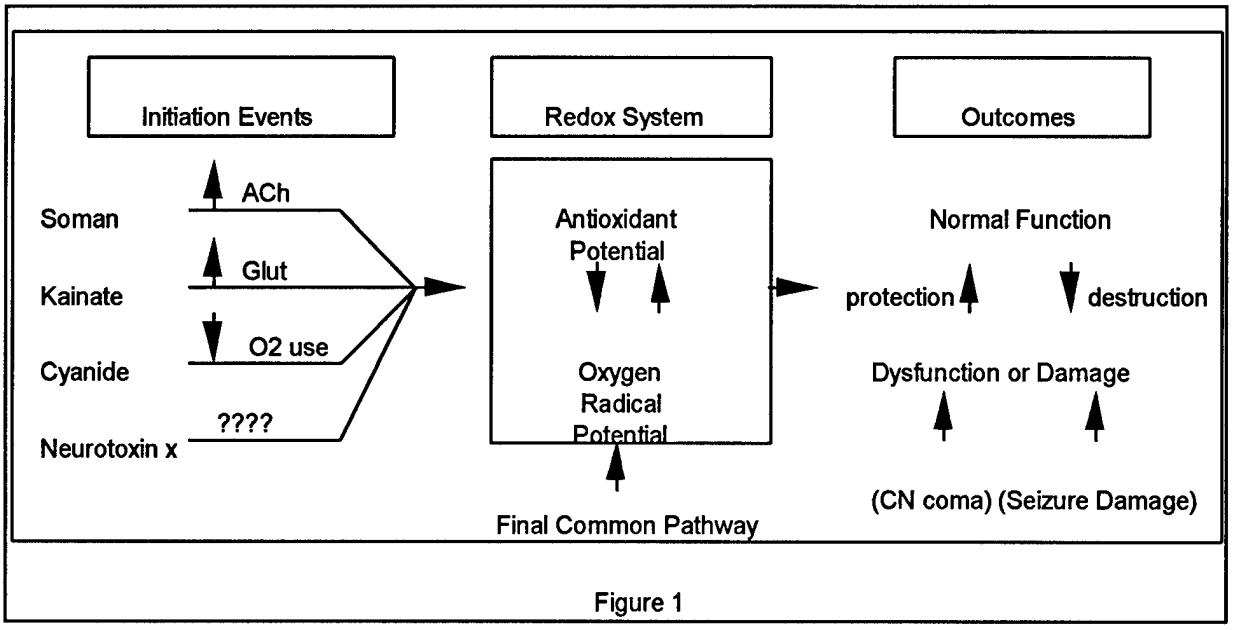

Figure 1

Therapeutic strategies targeted to free radicals as a final common pathway responsible for brain damage have the advantage that they may be given after the early events produced by a

neurotoxin. Further, they may be effective against brain injury caused by neurotoxins with diverse initial targets.

The problem investigated during this contract was to test the hypothesis that neurotoxins initiate a cascade of events that converge on redox mechanisms common to brain dysfunction or damage. This was accomplished according to the experiments outlined under Tasks 1-3:

Task 1. Detection of free radical biomarkers in the extracellular fluid via intracerebral microdialysis and in regional brain tissues.

Task 2. Detection of tissue biomarkers of free radical-induced gene expression responses by Northern and Western blot analysis.

Task 3. Pharmacological intervention of free radicals induced brain dysfunction.

BODY

EXPERIMENTAL METHODS

Animals: Adult male Wistar rats weighing 190-370 g were used. Food and water were provided *ad libitum*, and a 12-h light/dark cycle was maintained. All procedures involving animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC).

Agents: Sodium cyanide was obtained from Fisher Scientific Co. (Fairlawn, NJ). Cyanide was used in accordance with the security assurances required. All unused sodium cyanide solutions and contaminated material were alkalinized with KOH to prevent the escape of hydrogen cyanide (Robinson et al., 1984). Soman (1.9 mg/ml) from Chemical Systems Laboratory, Aberdeen Proving Ground, MD, was delivered on October 20, 1995. Kainic acid (Sigma Chemical Co., St. Louis, MO) was used as a seizuregenic surrogate of soman during periods that soman was not available in the laboratory.

Sodium cyanide exposure: Rats were given saline or NaCN by controlled intravenous infusion (20 µl/min; 4.5-5.0 mg/ml in 0.9% saline) via the femoral vein. The infusion was temporarily halted when the rat lost its righting reflex and resumed when righting occurred.

Soman exposure: Rats were given saline or a seizuregenic dose of soman (85-100 µg/kg; im) in 0.9% saline.

Kainic acid exposure: Rats were given Krebs Ringer bicarbonate (KRB) or seizuregenic dose of kainic acid (13-16 mg/kg; ip) in KRB.

Microdialysis probe assembly: Coaxial type probes were prepared from cellulose acetate membrane fibers from a kidney dialysis cartridge (O.D. 250 µm, Dow 50, MW cut-off

5000). Fused silica capillary of 155 µm O.D. was used as the inner tubing, and the rest of the liquid contact parts were made from 30 G Teflon tubing. The probe had a "Y" shaped assembly with fluid inlet and outlet tubings. Contact cement and clear epoxy resin were used to glue the parts together.

Perfusion apparatus: A Hamilton Microliter 1000 Series gas-tight syringe (total volume = 2.5 cc) with a Teflon-tipped plunger was used with a Harvard syringe infusion pump for constant flow delivery. A needle hub was constructed from a Teflon dowel rod (O.D. - 13 mm) to accommodate a standard syringe fitting (4 mm width) reduced over a 15 mm length to accommodate 20 gauge Teflon tubing (O.D. = 1.0 mm; I.D. = 0.86 mm). The 20 gauge Teflon tubing fit snugly over the probe inlet 24 gauge Teflon tubing; the seal was further improved at the junction by adding silastic tubing (Dow Corning; O.D. = 1.5 mm; I.D. = 1.0 mm) outside of the Teflon tubing.

Fiber implantation: Animals were anesthetized with pentobarbital (40 mg/kg, ip), prepared for surgery and placed in a stereotaxic apparatus. Anesthesia was supported with methoxyflurane. A hole was drilled in the appropriate place in the exposed skull to allow placement of the dialysis fiber. The stereotaxic coordinates from Paxinos and Watson (1982) relative to bregma were for right piriform cortex A: - 1.8,L: - 5.7, V: - 9.0 mm. In studies to measure nitric oxide, the fiber was implanted in the hippocampus A: - 5.6,L: - 5.0, V: - 7.0 mm.

In most cases, the experiments were started 24 hours after the fiber implantation. After the experiment was completed, fiber placement was verified histologically.

Microdialysis perfusion: All experiments were initiated 1 day after fiber implantation except for nitric oxide determinations and were done in unanesthetized freely moving rats. The

dialysis fiber input was connected to an infusion pump with Teflon tubing and a tubing fluid swivel. The fiber was perfused with Krebs Ringer bicarbonate (KRB, in mmol/L: NaCl, 122; KCl, 3; $MgSO_4$, 1.2; KH2PO_4, 0.4; $NaHCO_3$, 25 and $CaCl_2$, 1.2) or Ringer's solution (in mmol/L: NaCl, 148; KCl 3; $CaCl_2$, 2.5) at a flow rate of 2 - 4 µl/min with exceptions. Initially, the fiber was perfused with media alone for 2 hours and the samples discarded. The next 4 to 5 samples were used to determine basal levels of the substances to be analyzed. Samples were collected at intervals stated in figures.

Tissue extraction: Specified regional disected and weighed brain areas were added to two volumes of 0.3 M perchloric acid and two volumes of 0.2 M perchloric acid, 0.1 M Na2EDTA and 0.1 M $NaHSO_4$. The tissues were homogenized with a pestle and tube, filtered (0.2 µm) and analyzed for the specified analytes.

Ascorbate and urate analyses: HPLC/ECD Assay: Direct analysis of microdialysates for ascorbate and urate concentrations was done with HPLC electrochemical detection. The mobile phase consisted of sodium acetate (40 mM), dodecyltrimethylammonium chloride (1.5 mM), disodium ethylenediaminetetraacetic acid (0.54 mM) and methanol (7.5%), adjusted to pH 4.6 with glacial acetic acid. Flow rate was 0.8 µl/min. A reverse-phase 5 µm C-18 column, 250 x 4.6 mm, with a C-18 guard column was used for analyte separation with a BAS glassy carbon electrode detector set for 20 nA range. Potential was set at 700 mV.

Monoamine metabolites: 3,4-Dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindole acetic acid (5-HIAA) were quantitated by HPLC with electrochemical detection as described by Westerink et al. (1988). The mobile phase consisted of 0.025 M NaH2 PO_4, 0.15 M citric acid, 300 mg/L 1-octanesulfonic acid, sodium salt, 33.6

mg/L Na_2 EDTA and 22% methanol. Flow rate was 0.8 ml/min. A reverse phase 5 μm C-18 column, 250 x 4.6 mm, with a C-18 guard column was used for analyte separation with a ESA Coulchem II dual cell detector, the column was kept at 35°C. Cell 1 potential was 50 mV, cell 2 potential was 450 mV and the inline guard cell potential was set at 500 mV.

Salicylate and dihydroxybenzoic acids (DHBA): Salicylate, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) were quantitated by HPLC with coulometric detection. The mobil phase consisted of 100 mM NaH2 PO4 buffer, pH 2.5, 1.0% methanol and 0.25% isopropanol. Flow rate was 0.4 ml/min. A 150 x 4.6 mm ESA proprietary column was used for analyte separation with an ESA Coulchem II dual cell detector. Cell 1 potential was 275 mV, cell 2 potential was 700 mV and the inline guard cell potential was set at 500 mV.

Methods for nitric oxide detection with intracerebral microdialysis via hemoglobin trapping:

Equipment

A Shimadzu UV-160 spectrophotometer with a modified incident window height of 1.0 mm for microvolume measurement and a refrigerated water (0 °C) circulating thermobath (Haake, Germany) were used.

Hemoglobin stock

The stock solution was prepared according to Martin et al. (1985) and Murphy and Noack (1994). Briefly, 20 mg of reagent Hb (mostly MetHb) was dissolved in 2.0 mL Krebs Ringer bicarbonate in a 20 mL Erlenmeyer flask, and 5 mg of sodium dithionite was added to reduce Fe(III) to Fe(II). A stream of oxygen was blown gently into the flask for about 10 min with

occasional stirring to saturate the hemoglobin and remove excess dithionite. The resulting solution is then diluted to 6.0 mL with Krebs Ringer bicarbonate, and the excess inorganic sulfur compounds from dithionite were removed by dialyzing against Krebs buffer at 4 °C for 3 h. This stock solution was then dispensed into 100 μL aliquots in microcentrifuge tubes and stored at -75 °C. The stock solution had an absorption peak at approximately 414.5 nm, indicating a Hb content of ~95%. The concentration of hemoglobin is expressed in terms of μmol/L of hemes, which is four times that of the Hb protein concentration.

Spectroscopic measurement of hemoglobin

Cuvette of 0.7 mL capacity was cleaned with deionized distilled water (metals removed with cation resin) at least five times and blown dry with a constant flow of either nitrogen or helium gas. The inert gas flow was maintained in the cuvette at all times between measurements to ensure that the cell was free from dust and chemical contamination. Disposable pipettes were used to deliver 60 μL Hb solution to the cuvette and the spectrum was measured between 390 and 430 nm wavelength.

Data processing

The spectral data were either transferred to a Macintosh computer for further treatment or directly treated using the spectrum subtraction function versus a common reference spectrum of the initial Hb solution representing the background. The absorbance at 411 nm in the resulting spectrum was taken as the reference zero and a new zero absorbance line was drawn through this point. The spectrum was then enlarged on photocopy paper and the areas that represented the increase in MetHb and decrease in Hb were cut and weighed. The paper weight was proportional to the concentration of NO.

Microdialysis setup

A syringe pump (Harvard Apparatus) was used to deliver Krebs Ringer bicarbonate that contained 10 μM Hb hemes. The Hb was freshly diluted with 4 °C Krebs from the stock solution and loaded into a 5 mL gas tight Pyrex Glass syringe (Hamilton). Approximately 3-4 mL solution was used for each experiment. The liquid delivery system was protected from light and enclosed in a flexible socket cooling system which was circulated with 0 oC water. The perfusate delivery line was first flushed for 20 minutes at 20 μL/min and then a constant rate of 4 μL/min was maintained for NO detection either in buffer or in rats.

The last 15 cm (anesthetized rats) or 45 cm (awake rats) of inlet 30 G Teflon tubing was exposed to room temperature prior to connection to the microdialysis probe. Using a thermocouple, the inlet perfusion media was calculated to have warmed to 88% of room temperature after passing through 15 cm of room temperature exposed tubing. Therefore, the temperature of the perfusion media at the dialysis membrane should be no different than in routine microdialysis studies. The cooling of the syringe and early segments of the inlet tubing markedly reduced autoxidation of Hb. Cooling may not be necessary if freshly prepared rat Hb is used (Dr. A. Balcioglu; personal communication).

Brain microdialysis in rats

Wistar rats (12-16 weeks; 300-400 g) were anesthetized with chloral hydrate (7% in saline, ip) and the probe was implanted in the hippocampus stereotaxically using coordinates from Paxinos and Watson (1982) (5.6 mm posterior and 5 mm lateral to bregma and 7.0 mm below the dura). The probe was then fixed to the skull with a drop of commercial super glue followed by abundant denture resin. The fiber in the tissue was first equilibrated with the Hb Krebs Ringer

bicarbonate for about 60 minutes at a flow rate of 4 μL/min. Aliquots of the perfusate were then collected into microcentrifuge tubes and the spectra measured every 15 minutes (60 μL/sample). The spectrum of the second sample was usually saved on channel 2 on the instrument RAM to serve as the reference. Subsequent spectra were saved on channel 1 and each was subtracted from channel 2 and printed out. Such treatment gave the net change in the amount of Hb converted to MetHb relative to the common reference (channel 2). The printouts were then processed as described above. Numerical spectrum data were also printed for each sample for further treatment.

For experiments with awake rats, the probe was implanted under anesthesia. Twenty-four hours later, the probe was connected to the fluid delivery system through a fluid swivel. After the experiment was completed, fiber placement was verified histologically.

Nitrite assay:

The UV method uses 2,4-dinitrophenylhydrazine which reacts with nitrite under acidic conditions to form an azide. The analytical LC method is a modification of a preparatory LC method described by Kieber and Seaton (1995). Although the original method declares a limit of detection of 0.1 nM for natural waters, the method also had to overload the column with 1000 μl of sample. One of the difficulties with microdialysis sampling are the low sample volumes that are obtained typically in the 10-50 μl range. Because of the sample limitations in microdialysis, the detection limit of 0.1 nM may not be attainable. However, we have achieved a limit of detection of near 50 nM with an analytical column (4.6 mm x 150 mm) with 20 μl sample injections. Chromatographic theory predicts that using a smaller i.d. column should increase the sensitivity (defined as the change in response over the change in concentration) of the method and the limit

of detection of the method because of the reduction of dilution effects.

"Catalytic" iron assay: Samples (100-200 μg) were scraped from 20 μm brain sections at specified regions, sonicated for 30 minutes in 200 μL of 0.5 mM EDTA and assayed. The assay for "catalytic" iron, developed by one of us (S.R.N.), is an iron redox-cycling fluorometric assay (manuscript in preparation).

Tissue glutathione (GSH) assay: Tissue GSH in selected brain regions, expressed in μmole/gram of tissue, was determined by L.K. Klaidman (Univ. Southern California, Los Angeles, CA) as previously described (Adams et. al., 1983, 1993).

Methods for gene expression studies:

Kainic acid-treatment

Rats were injected with Krebs-Ringer bicarbonate (KRB) solution (controls; 1 ml/kg, ip) or kainic acid (KA; 14 mg/kg, ip) dissolved in KRB (14 mg/ml). KA was obtained from Sigma (St. Louis, MO). Rats with extensive tonic-clonic seizures ("responders") were used in the time course study for mRNA analysis. At time points of 0 (control), 1, 2, 4, 8, 24 and 120 hours after injection, animals (N=6 per group; except N=4 in 120 hour group) were anesthetized with halothane and decapitated, the brains quickly removed and the piriform cortex, frontal cortex and cerebellum dissected out and quick-frozen in 2-methyl butane at -70°C. In addition, groups of rats that did not have seizures after injection of KA were sacrificed at 4 (N=6), 8 (N=2) and 24 (N=3) hours and were labeled "non-responders". Brain regions from all rats in each group were pooled for RNA extraction.

Lipopolysaccharide (LPS) treatment

LPS from E. Coli 011:B4 (Sigma Chemical Co.) was prepared as a 1 mg/ml suspension in

sterile saline and bath sonicated for 4 min immediately before injection. A rat was injected with LPS (0.4 mg/kg, ip), and after 1 hour the rat was sacrificed by CO_2 asphyxiation and a portion of the liver removed and rinsed with saline. RNA was prepared as described below.

Isolation of total RNA

RNA was prepared by an initial guanidine thiocyanate (GTC)/phenol/chloroform extraction as described by Chomczynski et al. (1987) followed by sodium dodecyl sulfate (SDS)/phenol/chloroform extractions as described by Andrews et al. (1987). Briefly, dissected brain regions that had been frozen in 2-methyl butane and stored at -70°C, were homogenized with a Polytron (Brinkman Inst.) in a solution containing 4 M GTC, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol for 15 sec at the highest setting. Sodium acetate (1/10 volume, 4 M at pH 4) and an equal volume of phenol (water saturated) were immediately added, and the homogenization continued for 20 sec at highest speed. The aqueous and organic phases were separated by the addition of 1/10 volume of chloroform followed by centrifugation at 8,000 g for 10 min at 4°C. The aqueous phase was recovered and RNA precipitated by the addition of an equal volume of 2-propanol. After at least 2 hours at -20°C, the RNA precipitate was collected by centrifugation at 8,000 g for 10 min at 4°C. The precipitate was redissolved in SDS buffer (0.5% SDS, 25 mM EDTA, 75 mM NaCl, pH 8), extracted with phenol (saturated with SDS buffer) and then with phenol/chloroform:isoamyl alcohol (24:1 v/v). RNA was precipitated from the aqueous phase with 3 M ammonium acetate as described (Andrews et al., 1987). The RNA pellet was dissolved in water and reprecipitated with ethanol.

Northern blot hybridization

RNA was denatured for 5 min at 65°C in a solution containing MOPS buffer (20 mM

MOPS, 5 mM sodium acetate, 1 mM EDTA pH 7), 50% formamide and 2.2 M formaldehyde. Denatured RNA (2 g in 5 l) was size separated by electrophoresis in a 1% agarose gel containing 1X MOPS buffer and 2.2 M formaldehyde as described (Andrews et al., 1991), and transferred to Nytran membranes (Schleicher and Schuell, Keene, NJ) in the presence of 20X SSC (3 M sodium chloride and 0.3 M sodium citrate, pH 7). RNA was cross-linked to membranes by UV-irradiation (Stratalinker, Spectronics Corp.). Northern blots were prehybridized, hybridized and washed as described (Andrews et al., 1991), and hybrids were detected by autoradiography at -70°C with intensifying screens. In all experiments, duplicate gels were stained with acridine orange to verify integrity and equal loading of RNA. After autoradiography, membranes were stripped of probe by heating at 100°C for 10 min in a large volume of 0.02X SSC, 0.1% SDS. Successful removal of the probe was monitored by autoradiography for 18 hours. After prehybridization, membranes were rehybridized with each successive probe. In some experiments, membranes were subjected to radioimage quantitation using the radioanalytic image system (Ambis Systems Inc.).

Hybridization probes

cDNA clones obtained as described below were inserted into pGEM7Zf(-) vectors (Promega Biotec.) and used as templates for the synthesis of 32P-labeled cRNA probes as described by Melton et al. (1984) . Probes had specific activities of about 2 x 109 dpm/ g. The cDNA for mouse metallothionein-1 (MT-1) was provided by Dr. Richard Palmiter (University of Washington, Seattle, WA). The cDNA for mouse heat shock protein-70 (HSP-70) was provided by Dr. Richard Morimoto (Northwestern University, Evanston, IL). The cDNA for rat heme oxygenase-1 (HO-1) was provided by Ann Smith (University of Missouri, Kansas City, MO).

The cDNA for rat glutathione-S-transferase ya subunit (GSTya) was provided by Dr. Cecil Pickett (Merck Frosst Centre for Therapeutic Research, Quebec, Canada). A cDNA for rat glutathione peroxidase (GPx) was provided by Dr. Shinichi Yoshimura (Tokai University, Kanagawa, Japan). cDNA clones for rat Mn-SOD and rat CuZn-SOD (super oxide dismutase) were provided Dr. Harry S. Nick (University of Florida, Gainesville, Florida). A cDNA clones for mouse c-Fos was acquired through the American Type Tissue Culture Collection (No. 41041). A cDNA clone for mouse interleukin-1 (IL-1) was provided by Dr Robert Newton (The Dupont Merck Pharmaceutical Co., Glenolden, PA). A cDNA clone for mouse MT-III was generated by RT-PCR as described below.

Reverse transcriptase (RT)-PCR cloning of mouse MT-3 cDNA

RT-PCR conditions were essentially as described (Andrews et al., 1991). Oligonucleotide primers (shown below) were synthesized based the on the mouse MT-3 cDNA sequence (Palmiter et al., 1992).

5' CCGAATCCGTGTGCCAAGGACTGTGTGTGC 3'(sense)

5' CCGGATCCGACAACAGTTGTGCCCCACCAG 3'(antisense)

The sense strand primer extended from the wobble position of codon 44 through codon 51 and the antisense primer encompassed bases 267 to 289 in the 3' untranslated region. The 5' termini of these primers include restriction sites used in cloning and 2 terminal bases for stability.

The synthesis of single stranded mouse MT-3 cDNA used the antisense strand mMT-III oligonucleotide as a primer for reverse transcriptase and mouse brain poly(A+) RNA (1 g) as a template as described (Andrews et al., 1991). Reaction products (2 l) were amplified by PCR (Saki et al., 1988) for 35 cycles using the cycle parameters described (Andrews et al., 1991).

The major reaction product (153 bp) was cleaved with EcoRI and BamHI and cloned between those sites in pGem7Zf(-) (Promega Biotech.). The nucleotide sequence was determined on both strands by the dideoxynucleotide chain-termination method (Sanger et al., 1977) and the Sequenase version 2.0 (United States Biochemicals) and was identical to the published sequence of MT-III (Palmiter et al., 1992).

Preparation of poly(A+) RNA

Total RNA (2 mg in 0.5 ml) was denatured for 5 min at 65°C and an equal volume of 2X binding buffer (20 mM Tris/HCl, 1 M LiCl, 2 mM EDTA, and 0.2% SDS, pH 7.5) was added. This mixture was applied to a column containing 10 mg of oligo dT cellulose (Collaborative Biochemicals) that had been extensively washed with elution buffer (10 mM Tris/HCl, 1 mm EDTA, and 0.1% SDS, pH 7.5) and equilibrated with at least 20 volumes of 1X binding buffer. After the flow through had been reapplied to the column, the column was washed with 10 volumes of 1X binding buffer, 5 volumes of wash buffer (10 mM Tris/HCl, 150 mM LiCl, 1 mM EDTA, and 0.1% SDS, pH 7.5), and eluted with 3 volumes of elution buffer. The eluate was collected, adjusted to 0.3 M ammonium acetate and precipitated with 2 volumes of ethanol. After centrifugation, the pellet was dissolved in 30 l of water that had been filtered and then autoclaved.

Western Blotting

Brain tissue homogenates were prepared and clarified according to Mizzen, et al (1996). Total protein was determined by the Lowry method (Sigma, St. Louis, MO). Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 18% gels (MT-1,2) and 15% gels (HO-1) (Laemmli, 1970). For MT-1,2, electrophoretic transfer

of the separated protein to nitrocellulose membranes at 40 V for one hour in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), 2 mM $CaCl_2$ and 10% v/v methanol, pH = 10.8 and subsequent glutaraldehyde treatment were performed according to Mizzen, et al (1996). For HO-1, transfer was performed similarly except at 100 V for one hour in 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH = 8.3.

Membranes were blocked with 3% BSA in TBS (20 mM Tris, 500 mM NaCl, pH = 7.4) for 2 hours at room temperature before incubation overnight with primary antibody [mouse monoclonal antibody to polymerized equine renal MT-1,2 (Dako, Cardinteria, CA) or a rabbit polyclonal antibody to HO-1 (Stressgen, Victoria, BC, Canada)] at a 1:1000 dilution in TBS. Membranes were washed with TBST (20 mM Tris, 500 mM NaCl, 0.2% Tween-20, pH = 7.4). Secondary antibody incubation was for four hours with either goat anti-mouse (MT-1,2) or goat anti-rabbit (HO-1) IgG conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA). Membranes were washed again with TBST and the Immun-Star Chemiluminescent Protein Detection System (Bio-Rad, Hercules, CA) was used to develop blots, with the signal captured on X-ray film.

RESULTS AND DISCUSSION

Task 1. Studies on brain extracellular fluid and specified regional brain tissues: Results from previous studies in our laboratory indicate that ascorbate levels in brain extracellular fluid sampled by microdialysis increase rapidly after brain insults (e.g., trauma, seizures). The increase in ascorbate is independent of brain activity, whereas urate levels increase more slowly, and the increase in urate levels is at least in part dependent on brain activity. Moreover, hydrogen

peroxide can be measured in microdialysis perfusate samples and the amount of hydrogen peroxide increases as the perfusate sample sits on the bench-top. Since it was clear from previous studies in our laboratory that brain extracellular fluid has both pro-oxidant and antioxidant effects, we had Maria Romanas, M.D./Ph.D. candidate, evaluate the pro-oxidant and antioxidate nature of extracellular fluid to complement the studies under investigation in this contract.

Her work was based on the proposal that hydroxyl radicals generated by ascorbate-driven Fenton-like reactions in brain extracellular fluid contribute to the pathology of acute brain insults. The hypothesis is that the antioxidant capacity of brain extracellular fluid is sufficient to block ascorbate-driven Fenton-like salicylate hydroxylation. Salicylate hydroxylation is used as a selective indicator for hydroxyl radical levels. The addition of hydroxyl radicals to salicylate yields the 2,3 and 2,5 isomers of dihydroxybenzoic acid (2,3- and 2,5-DHBA). A fluorescence assay was developed to follow the time course of 2,5-DHBA formation during incubations of salicylate, ascorbate, a metal catalyst, and H_20_2 in Chelex-treated phosphate buffer or in human cerebrospinal fluid.

Salicylate hydroxylation occurs in two phases: a rapid, oxygen-dependent phase and a slow, less oxygen-dependent phase. Initially, electron transfer in an ascorbate-metal-0_2 complex occurs rapidly to reduce another metal ion that reacts with H_20_2 in a Fenton-like manner to generate hydroxyl radicals. During the slow phase, electron transfer within an ascorbate-metal-H_20_2 complex is slow; however, the complex can decompose to yield hydroxyl radicals.

The activities of several metal forms for catalyzing salicylate hydroxylation were compared at normal and acidic pH (7.4, 6.4, 5.4). Copper (Cu^{2+}), hematin, and hemoglobin had

the highest activities of the metal catalysts used. Chelated iron (Fe^{3+}-EDTA and Fe^{3+}-citrate) had more activity than "free" iron (Fe^{3+}). The order of activity seen for these catalysts may correspond to their ability to bind dioxygen. With increasing catalyst concentration or with increasing acidity, the rate of the rapid phase was enhanced, but the slow phase dominated earlier. Therefore, acidosis following acute brain insults probably enhances the generation of hydroxyl radicals by ascorbate-driven Fenton-like reactions, especially if the metal is copper or heme-iron.

Several-fold increases in brain extracellular fluid ascorbate and urate occur following acute brain insults; therefore, the effects of a wide concentration range of ascorbate and urate were tested. With increasing ascorbate, initial rates of salicylate hydroxylation were enhanced up to a certain point. The slower phase of salicylate hydroxylation dominated earlier and became more prominent with increasing ascorbate. Urate had no significant effect at concentrations normally found in brain ECF. However, higher concentrations of urate inhibited salicylate hydroxylation while simultaneously increasing ascorbate oxidation. The factors that determine whether ascorbate or urate will have a pro-oxidant or an antioxidant effect in a particular system are: (1) the relative concentration of metal, (2) the concentrations of oxygen and H202, and (3) the manner in which the critical substrate is oxidized.

Brain extracellular fluid contains very little protein but contains high concentrations of water-soluble molecules. Lactate levels, in particular, increase in brain ECF following acute insults. The effects of glucose, lactate, citrate, and inositol were tested at concentrations relevant to brain ECF. Lactate, glucose, and inositol inhibited salicylate hydroxylation in a concentration-dependent manner. Citrate inhibited, enhanced, or had no effect on systems catalyzed by copper, iron, and iron EDTA respectively. Although glucose, lactate, citrate, and

inositol are not "antioxidants" by definition, they may have "antioxidant effects" on hydroxyl radical generation and action in brain extracellular fluid by virtue of their high concentrations.

Human cerebrospinal fluid had a great capacity to decrease hydroxyl radical levels in systems containing ascorbate, metals, and H_20_2. The inhibition of ascorbate driven salicylate hydroxylation probably occurs by hydroxyl radical scavenging and by metal chelation which are both mediated by high concentrations of small water-soluble molecules (e.g. urate, glucose, lactate, inositol, and citrate) with a molecular weight less than 5000 in human CSF. This indicates that hydroxyl radicals are too reactive to be important oxidizing species in brain extracellular fluid.

Metal delocalization and H_20_2 generation following acute brain insults might also result in the formation of "activated ascorbate", a ternary complex ascorbate metal-H202, which may be more important than hydroxyl radicals in mediating the delayed pathology of acute brain insults. This complex may mediate oxidations independent of hydroxyl radicals analogous to "activated bleomycin", a bleomycin metal-H_20_2 complex.

From these studies, manuscripts are in preparation. These studies clearly delineate the complex nature of redox chemistry in biological fluids. We conclude that hydroxy radicals that form in biological fluids are rapidly inactivated, whereas ascorbate/metal/peroxide complexes are considerably less reactive and can diffuse to critical targets causing site specific damage.

To study changes in ascorbate and urate in brain extracellular fluid during cyanide exposure, a microdialysis fiber was implanted into the right piriform cortex and perfusate was collected before, during and after NaCN exposure. NaCN exposure was a described in the results. Alternate 15 minute samples were analyzed for ascorbate/urate (Figure 2) and monoamine

metabolites (Figure 3).

To study changes associated with soman, induced seizures, a microdialysis fiber was implanted in the right piriform cortex. The following day, basal perfusion levels were established, salicylate (100 mg/kg, ip) was injected, samples were collected for 1 hour, and then a seizuregenic dose of soman (100 μg/kg; im) was injected. Alternate 10 minute samples were analyzed for ascorbate/urate (Figure 4), salicylate (Figure 5), dihydroxybenzoic acids (Figure 6) and monoamine metabolites (Figure 7). These studies clearly indicate that there are changes in redox active substances in brain extracellular fluid occur after exposure to either cyanide or soman. The dramatic increase in monoamine metabolites suggests that monoamine turnover has increased and/or that the active transport of monoamine metabolites from the brain were impaired.

Nitric oxide (NO) is involved in many physiological, immunological and neurological processes such as neurotransmission, memory, brain injury, blood pressure regulation and blood clotting (Ignarro et al.,1987, 1993; Ignarro 1990; Feldman et al., 1993; Lipton et al., 1994; Stamler et al., 1992). However, its precise role in these processes awaits improved methods in NO detection. Developing a method for in vivo NO sampling and detection is challenging because NO has a short half-life (3-5 seconds in physiological environments) and occurs at sub-micromolar concentrations (Ignarro, 1990; Ignarro et al., 1993).

Microsensor and microdialysis probes provide viable approaches for direct in vivo measurements of NO (Malinski and Taha, 1993; Miyoshi et al., 1994; Ichimori et al., 1994; Mitsuhata et al., 1994). Nitric oxide has been detected in the brain by the microdialysis

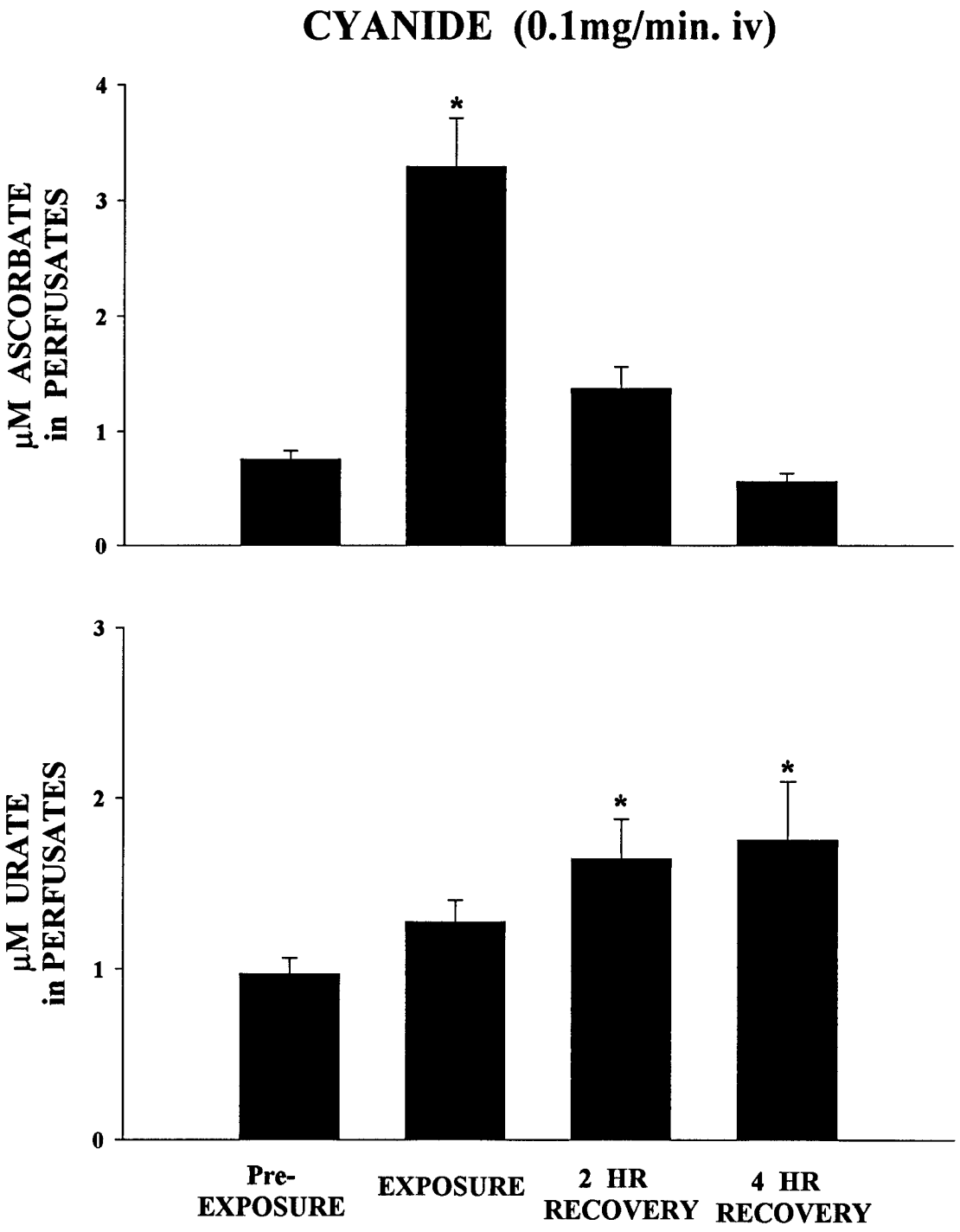

Figure 2. Concentrations of ascorbate (μM) and urate (μM) in microdialysis perfusates before, during and after cyanide exposure. A microdialysis fiber was implanted into the right piriform cortex and NaCN was given by intravenous infusion as stated in methods. Alternate 15 minute samples were analyzed and results were pooled over the periods indicated (N= 8). * = significantly different from pre-exposure at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

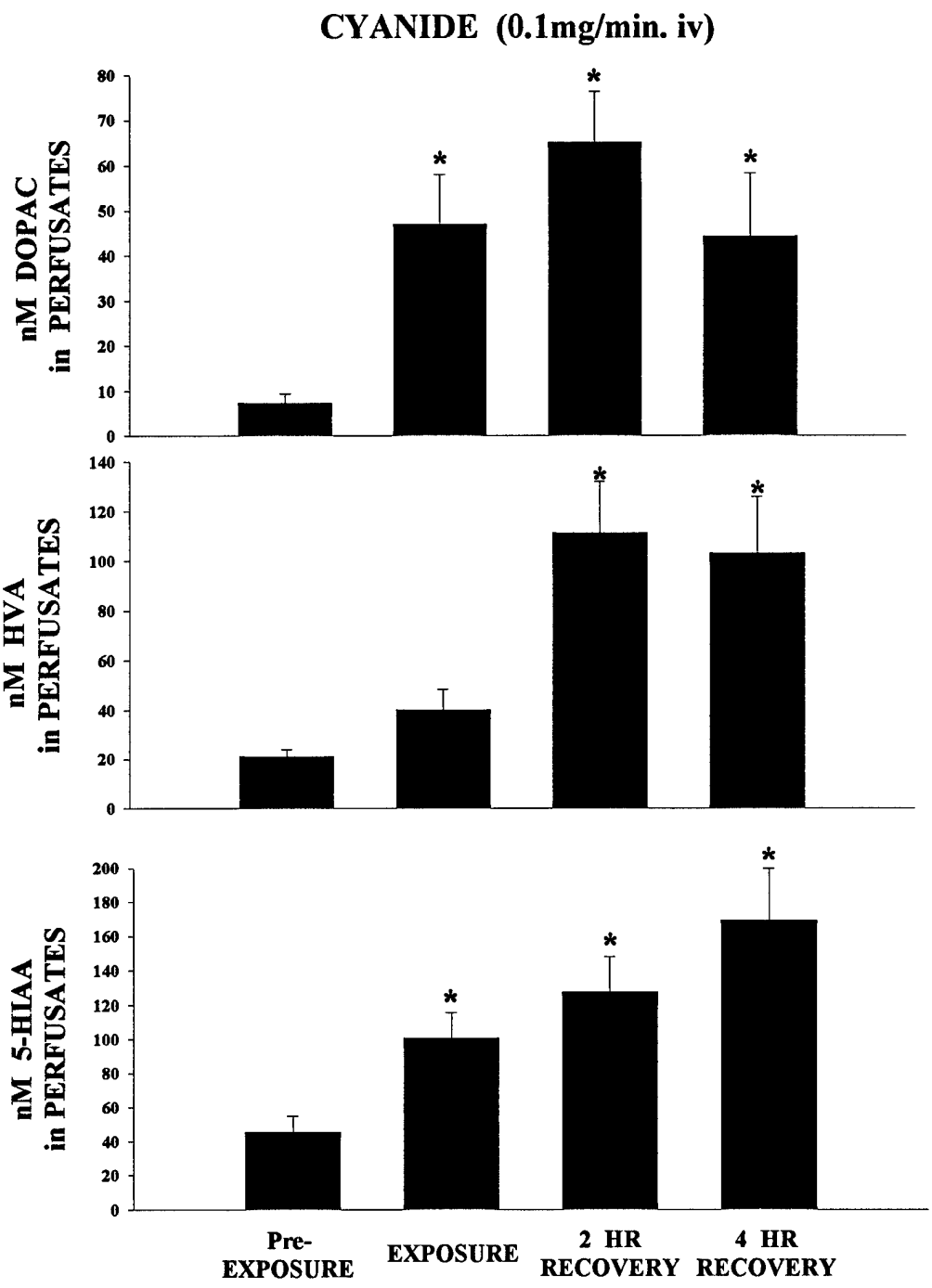

Figure 3. Concentrations of DOPAC (nM), HVA (nM) AND 5-HIAA (nM) in microdialysis perfusates before, during and after cyanide exposure. A microdialysis fiber was implanted into the right piriform cortex and NaCN or saline was given by intravenous infusion as stated in methods. Alternate 15 minute samples were analyzed and results were pooled over the periods indicated (N=5). * = significantly different from pre-exposure at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

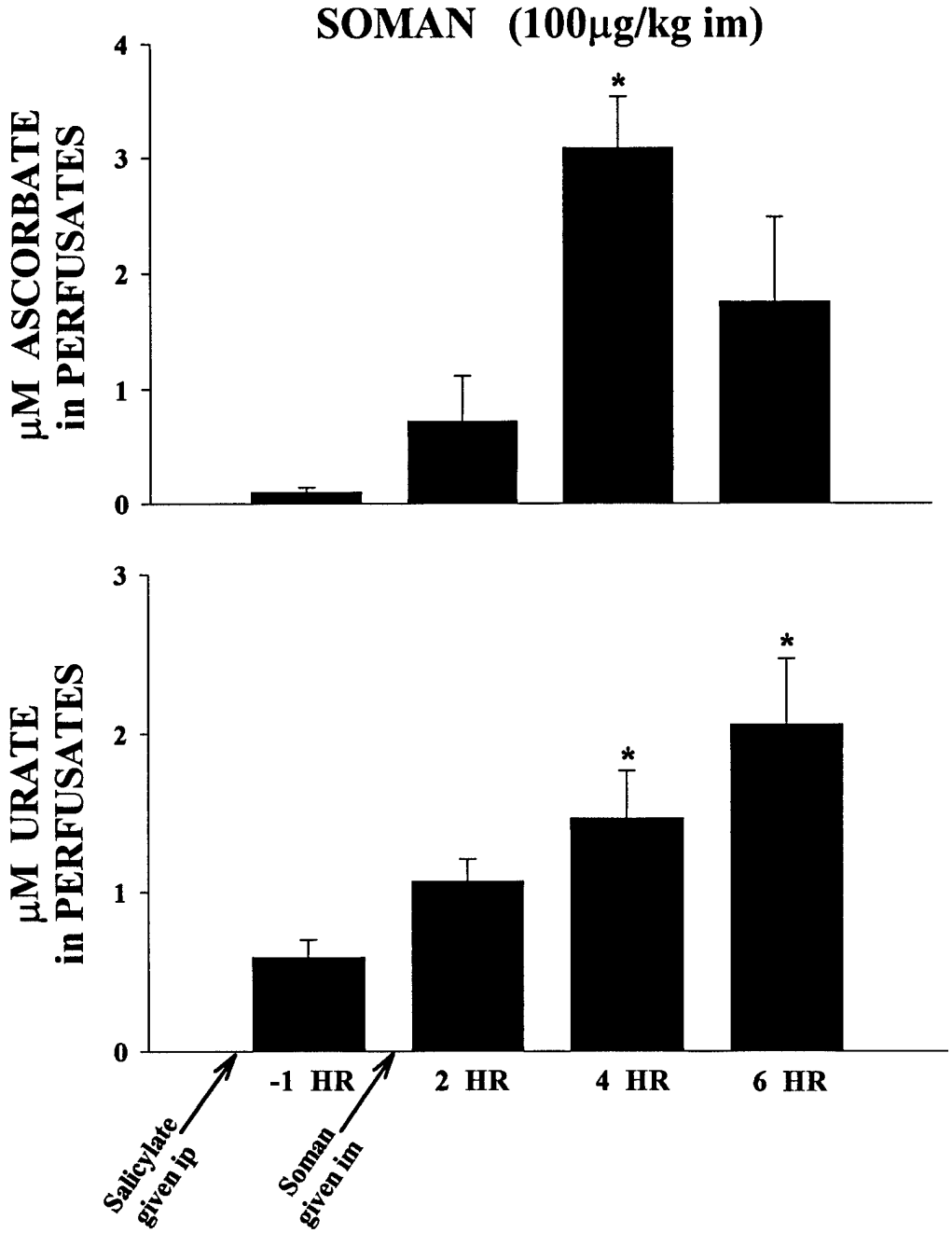

Figure 4. Concentrations of ascorbate (µM) and urate (µM) in microdialysis perfusates after salicylate (100 mg/kg; ip) and soman injection (100 µg/kg; im). A microdialysis fiber was placed in the right piriform cortex and salicylate and soman were given as stated in methods. All rats had typical soman-induced seizures. Alternate 10 minute samples were analyzed and results were pooled at times indicated (N=3). * = significantly different from -1HR using one-way ANOVA and Dunnett's post-hoc test.

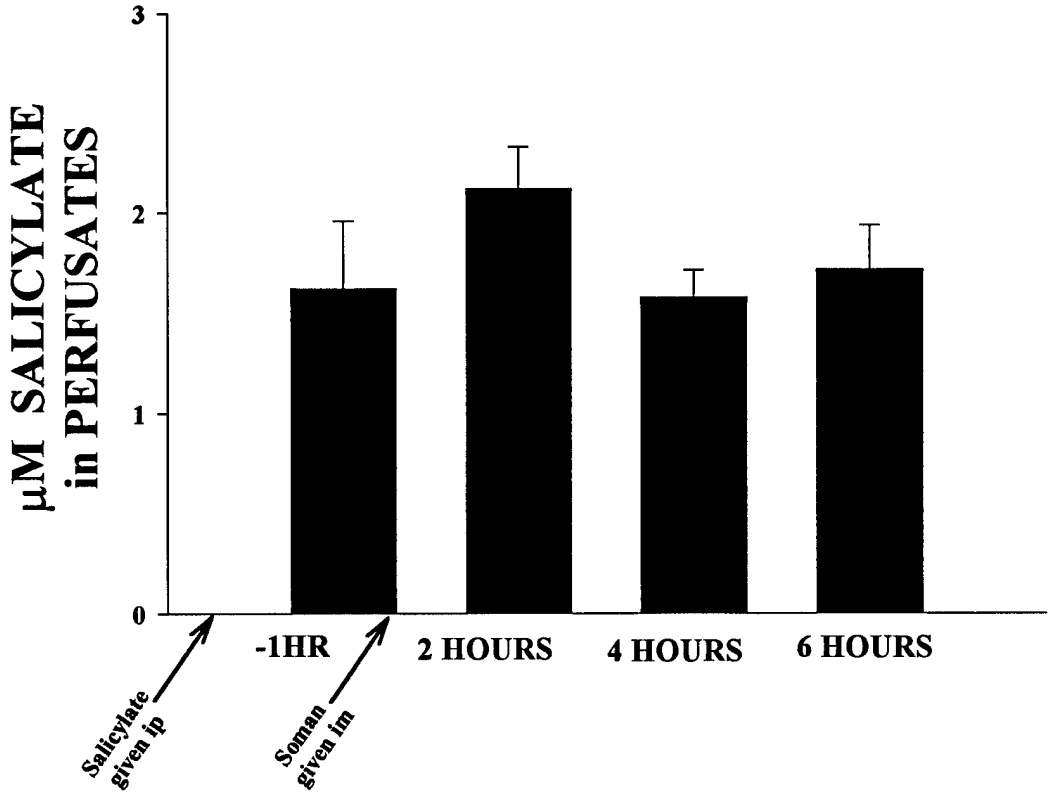

Figure 5. Concentrations of salicylate (μM) in microdialysis perfusates after salicylate (100 mg/kg;ip) and soman (100 μg/kg; im) injections. A microdialysis fiber was placed in the right piriform cortex and salicylate and soman were given as stated in methods. All rats had typical soman-induced seizures. Alternate 10 minute samples were analyzed and results were pooled at times indicated. N=3. No sigificant differences from -1HR were found using one-way ANOVA.

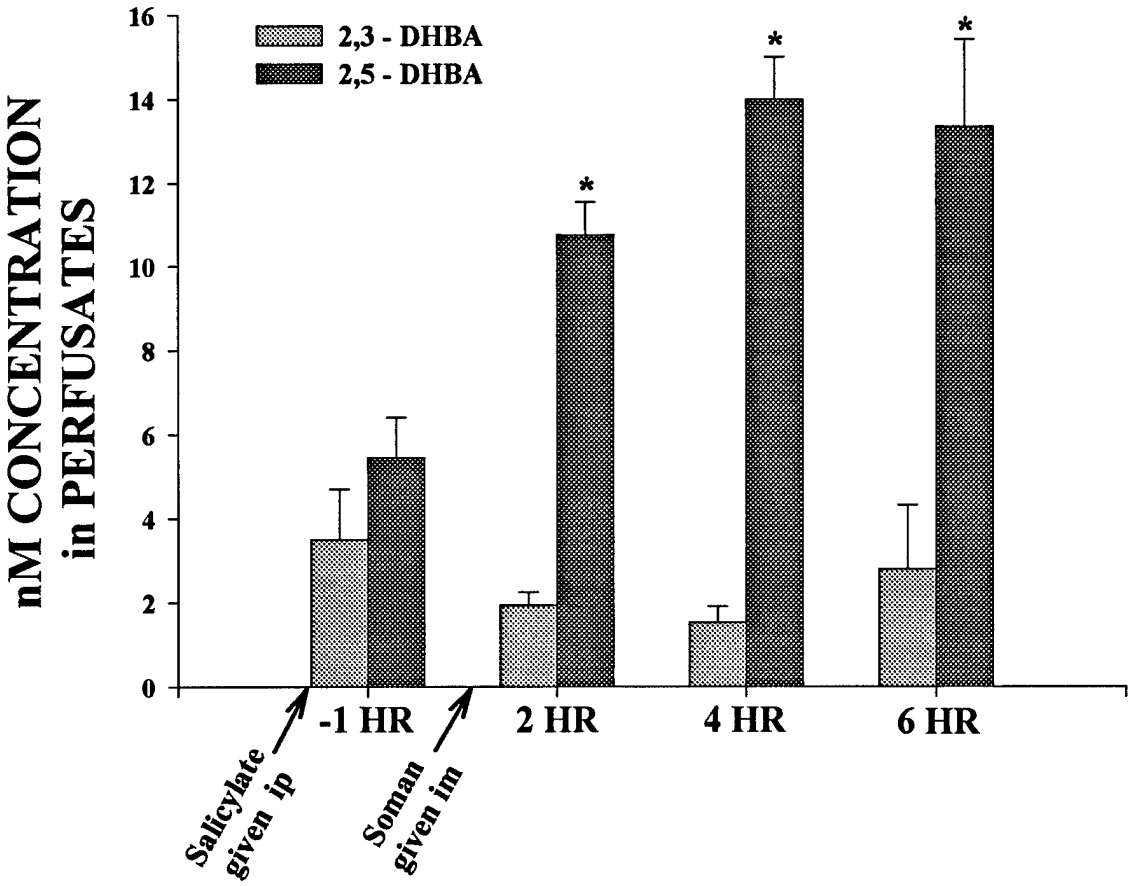

Figure 6. Concentrations of 2,3-DHBA (nM) and 2,5-DHBA (nM) in microdialysis perfusates after salicylate (100 mg/kg; ip) and soman injection (100μg/kg; im). A microdialysis fiber was placed in the right piriform cortex and salicylate and soman were given as stated in methods. All rats had typical soman-induced seizures. Alternate 10 minute samples were analyzed and results were pooled at times indicated. N=3. * = significantly different from -1HR at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

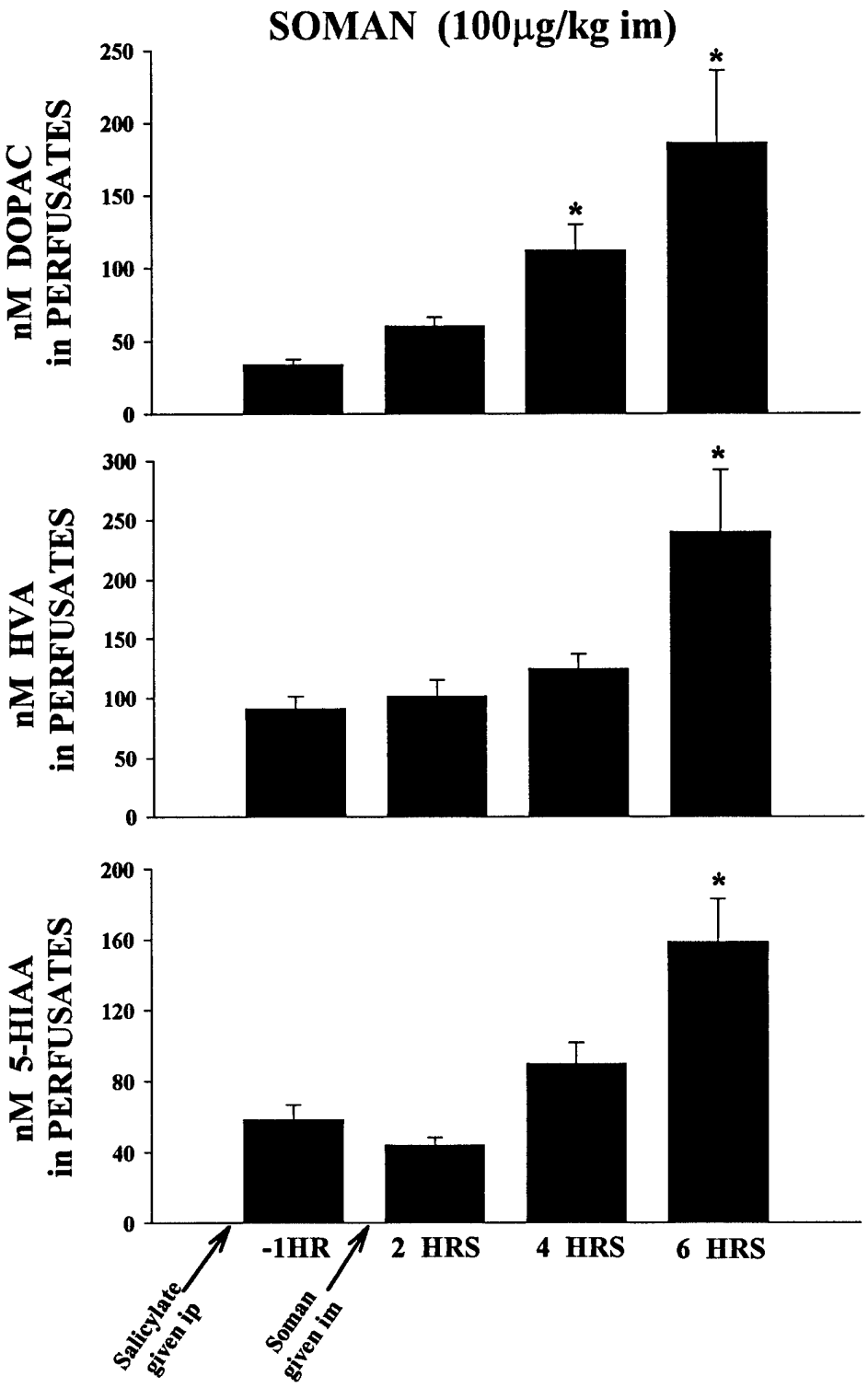

Figure 7. Concentrations of DOPAC (nM), HVA (nM) and 5-HIAA (nM) in microdialysis perfusates after salicylate (100 mg/kg; ip) and soman (100 μg/kg; im) injections. A microdialysis fiber was placed in the right piriform cortex and salicylate and soman were given as stated in methods. All rats had typical soman-induced seizures. Alternate 10 minute samples were analyzed and results were pooled at times indicated. N=3. * = significantly different from -1HR at $P<0.05$ using one-way ANOVA and Dunnett's post-hoc test.

hemoglobin (Hb) trapping method in anesthetized rats (Balcioglu and Maher, 1993, 1994). Hemoglobin (Hb) is an effective scavenger for NO (Murphy and Noack, 1994; Feelisch and Noack, 1987) with the affinity of NO for Hb being 105-6 times higher than that of oxygen, and after NO binds, hemoglobin (Fe(II)) is quantitatively converted to methemoglobin (Fe(III); MetHb), simultaneously releasing nitrate (Feelisch and Noack, 1987; Archer, 1993,). An analytical approach to measure NO concentration is based on a shift in the Soret band of the heme protein absorption spectra when Hb (max = 415 nm) is converted to MetHb (max = 406 nm). Nitric oxide can be quantified by measuring the decrease in Hb or increase in MetHb. This method is highly sensitive because of the high molar absorptivity of Hb (ϵ=1.3x105 M^{-1} cm^{-1}, Murphy and Noack, 1994); its sensitivity can be two orders of magnitude higher than the indirect methods for NO analysis such as measurement of nitrite and nitrate by the Griess assay or HPLC-conductivity detection (Green et al., 1982; Lippsmeyer et al., 1990; Misko et al., 1993; Luo et al., 1993; Shintani et al., 1994).

Several factors must be considered when using the hemoglobin-trapping technique to measure NO . First, reagents require a reasonable degree of chemical and thermal stability. Hemoglobin has the disadvantage of being vulnerable to degradation and the iron(II) is prone to oxidation by light or long contact time with the tubing surface. Second, an adequate and reliable data processing method is needed because absorbance measurements are limited (resolution and noise). Third, an appropriate calibration is needed to assure that NO is detected quantitatively because stoichiometric NO generating agents are unavailable. Fourth, it is essential to verify that NO can indeed diffuse through the fiber membrane to reach the dialysate. Finally, the method needs to be tested in vivo taking into account in vivo background fluctuations in order to

assess the sensitivity and detection limit.

To accomplish this goal, the hemoglobin trapping technique for nitric oxide detection with intracerebral microdialysis was done as described in methods. A manuscript entitled "Nitric oxide detection with intracerebral microdialysis: Important considerations in the application of the hemoglobin-trapping technique has been published: Journal of Neuroscience Methods 68: 165-173 (1996). The effects of seizuregenic dose of kainic acid on nitric oxide detection in brain extracellular fluid in anesthetized (Figure 8) and awake rats (Figure 9) are shown.

Although the purpose of the in vivo experiments was to test whether the Hb to MetHb method provides sufficient sensitivity to capture NO, it was also valuable to measure NO changes in the extracellular fluid of both anesthetized and awake rats injected with the excitotoxin KA. The profile of NO change in the hippocampus of anesthetized rats is basically in agreement with reported results (Balcioglu and Maher, 1993); the NO concentration returns to basal level two hours after KA administration. Further, inhibition of nitric oxide synthase by NG-nitro-L-arginine hydrochloride (L-NNA) completely abolishes the increase in NO concentration. This is an indication that the increase in Hb to MetHb conversion in the microdialysate is the result of direct NO reaction with Hb. Surprisingly, the changes in NO were similar in anesthetized and non-anesthetized rats. The apparent larger response in the anesthetized rats is due to the longer probes used in this group of rats. The increase in NO occurred at about anesthetized rats whereas the increase in NO occurred between 60-120 min in non-anesthetized rats and correlated with the onset of tonic-clonic seizures.

We and others have found that rats do not survive KA-induced seizures when both endothelial and neuronal nitric oxide synthase is inhibited (Tanaka et al., 1991; Zuo et al., 1992;

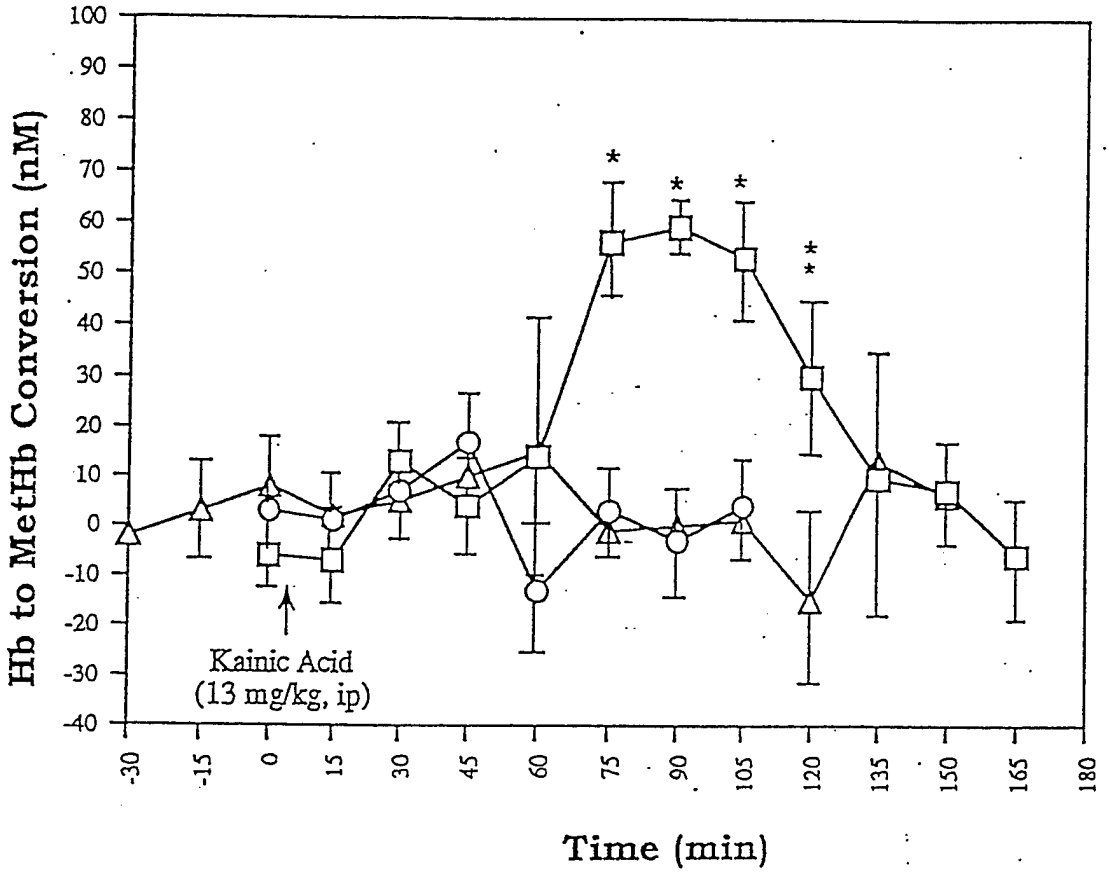

Figure 8. Measurement of $NO^{\cdot}$ change in microdialysates from rat brain. Error bars are ±SEM. Group A (-□-, n=4): Rats were treated with KA (13 mg/kg, ip). Group B (-□-, n=3): Rats were treated with N^{G}-nitro-L-arginine hydrochloride (L-NNA) (30 mg/kg, ip) 30 minutes before KA administration. Group C (-O-, n=3): Rats without KA and L-NNA treatment. (*) : $P < 0.01$ and (**): $P < 0.05$ vs control values at the same times. Rats were anesthetized with chloral hydrate. The probes were implanted in the hippocampus and were infused with 7 μM Hb heme in Krebs buffer at a flow rate of 4.0 μL/min. The length of the probe was 4.5 mm. Samples were collected every 15 minutes.

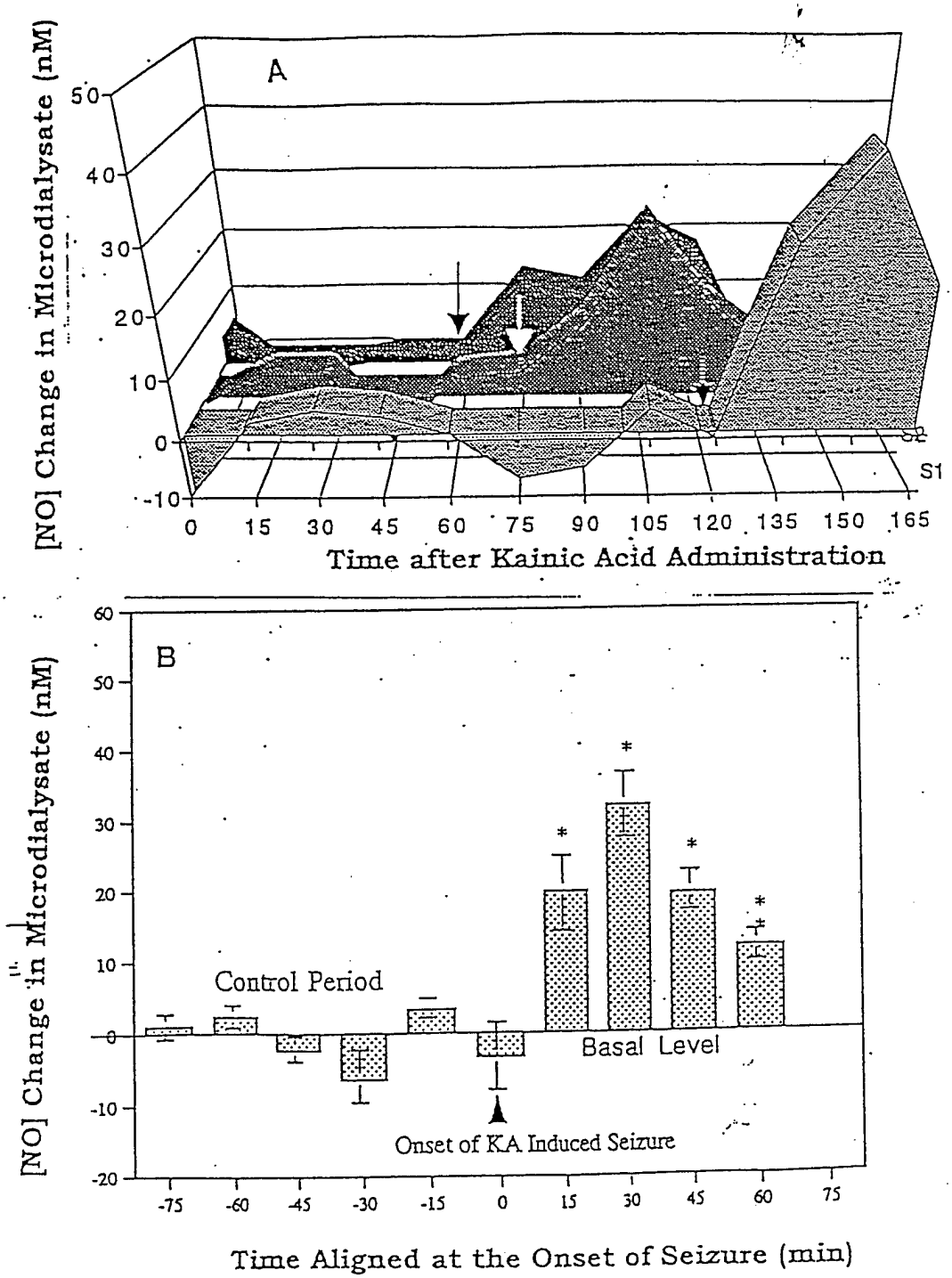

Figure 9. (A). Three individual experiments of NO˙ measurement in the microdialysates in awake rats aligned at the time of KA injection (13 mg/kg, ip at t=0). The length of the probe was 3.0 mm. The time between KA administration and the seizure varies significantly (60 min - 120 min). Arrows denote the onset of seizure. (B). NO˙ concentration in the microdialysates aligned at the onset of seizure. Error bars are ±SEM. The significance of the difference in the level of NO˙ due to seizure was compared to NO˙ level during the control period (before seizure). *. $P<0.01$; **. $P<0.05$.

Rigaud-Monnet et al, 1994). This is likely due to the fact that blockade of nitric oxide synthase inhibits hippocampal hyperemia in KA-induced seizures (Rigaud-Monnet et al, 1994). The time course profile of NO changes found during KA-induced seizures supports this notion (Figure 9). In contrast, the selective inhibition of neuronal nitric oxide synthase with 7-nitroindazole appears to attenuate KA-induced seizures and subsequent neurotoxicity (Mülsch et al., 1994). Studies on extracellular NO changes under different experimental paradigms are needed to clarify the complex role of NO in seizure activity, cerebral blood flow and brain damage associated with kainic acid induced seizures. (Rigaud-Monnet et al., 1995; Maggio et al., 1995; Przegalinski et al., 1994).

Although this method works to measure nitric oxide, it is difficult to carry out and will not work with cyanide, since cyanohemoglobin formation interferes with hemoglobin to methemoglobin determination.

The original contract stated that nitric oxide would be detected indirectly using the Griess reaction. Since NO reacts with O_2 and $O2^{-}$ in vivo to form NO_2^- and NO_3^-, selection of the Griess reaction was a viable first choice for analysis. The Griess reaction is a classic method for determining nitrite, NO_2^-, in solutions. The assay involves adding equal parts of nitrite, 0.1% N-(1-naphthyl ethylenediamine) and 1 % sulfanilic acid in a 5% solution of concentrated H_3PO_4. The detection limits claimed for this reaction range from 200 nM to 400 nM. NO_3^- must be reduced with a copper-coated cadmium wire or by using the enzyme nitrate reductase.

Although other groups have claimed to use the Griess reaction with microdialysis samples, we have found the Griess reaction to not be amenable for our detection needs of nitrite. The principal shortcoming of the method in this laboratory was its limit of detection, which was

determined to be 1 μM with the equipment available to us. Because we expect nitrite levels to be lower than 1 μM in microdialysis samples, an alternate method was needed. We have tried two alternate methods- a fluorescence method and a UV method.

We first tried a fluorescence assay that was a modification of a diazotization method described by Misko et al. (1993). This assay was initially performed in microtiter plates and read by a special fluorimeter. Since we do not have access to such equipment, a liquid chromatography (LC) method was prepared. The initial method used a pH of 7 in the mobile phase. The fluorescence maximum of the derivative of nitrite is between pH 9-11. This required the use of polymer column, which was ordered and took several weeks to arrive. Once the polymer column arrived and the mobile phase was adjusted to pH 9, a very large background was observed in the blanks with the derivitizing agent. The interference was not removed by using a different vendor's product. Another literature search brought to our attention a recent UV method.

This method was used as described in methods. A standard curve is shown in Figure 10. Since Dr. Julie Stenken, the postdoctoral fellow working on this problem accepted an academic position, we did not pursue this problem any further.

In order to better understand the changes in redox active compounds associated with cyanide and soman we designed experiments to study changes of key molecules in tissues. The first set of experiments were with cyanide. Rats were given intermittent cyanide exposure (0.1 mg/min via iv infusion for 90 minutes in order to keep rats from righting during this 90 minute period. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Rats were sacrificed 2 and 10 hours after cyanide fusion was initiated. Regional brain tissues were harvested, homogenized

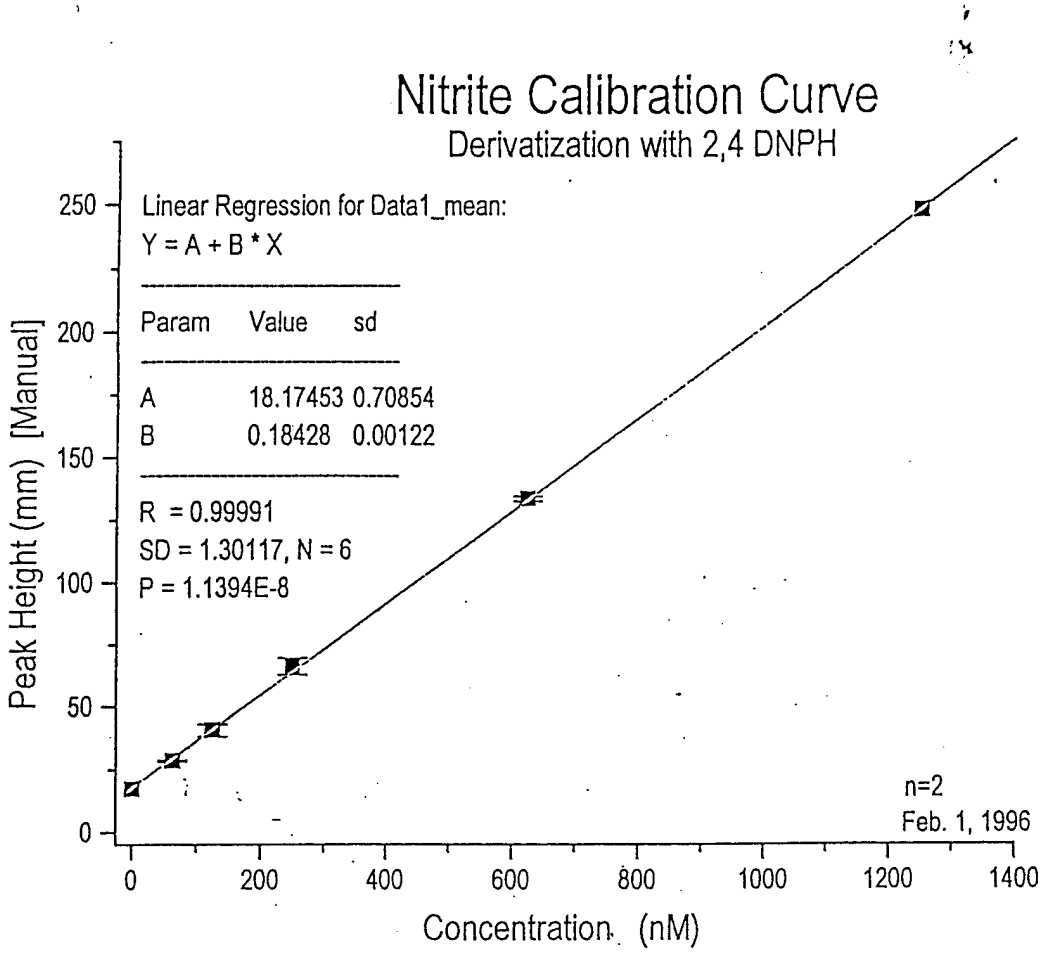

Figure 10. Nitrite calibration curve.

and analyzed for analytes and expressed as follows: Figure 11, urate in piriform, parietal and frontal corticies; Figure 12, urate in hippocampus and caudate; Figure 13, salicylate in piriform, parietal and frontal cortices; Figure 14, salicylate in hippocampus and caudate; Figure 15, 2,3-DHBA in piriform, parietal and frontal cortices; Figure 16, 2,3-DHBA in hippocampus and caudate; Figure 17, 2,5-DHBA in piriform, parietal and frontal cortices; Figure 18, 2,5-DHBA in hippocampus and caudate; Figure 19, DOPAC in piriform, parietal and frontal cortices; Figure 20, DOPAC in hippocampus and caudate; Figure 21, HVA in piriform, parietal and frontal cortices; Figure 22, HVA in hippocampus and caudate; Figure 23, 5-HIAA in piriform, parietal and frontal cortices; Figure 24, 5-HIAA, urate in hippocampus and caudate. This study did indicate that cyanide caused minor changes in redox active and monoamine metabolite analytes. However, the number of animals studies were small so it is difficult to make a definitive conclusion.

The second set of studies focused on the tissue changes associated with soman exposure. Rats were given a seizurogenic dose of soman (80-90 μg/kg; im) and salicylate (50 mg/kg; ip) was given 2.5 hours prior to sacrifice. Rats were sacrificed at 0, 2, 10 or 24 hours post soman. Regional brain tissues were harvested, homogenized and analyzed for analytes and results are expressed as shown: Figure 25, urate in piriform, parietal and frontal cortices; Figure 26, urate in hippocampus and caudate; Figure 27, salicylate in piriform, parietal and frontal cortices; Figure 28, salicylate in hippocampus and caudate; Figure 29, 2,3-DHBA in piriform, parietal and frontal cortices; Figure 30, 2,3-DHBA in hippocampus and caudate; Figure 31, 2,5-DHBA in piriform, parietal and frontal cortices; Figure 32, 2,5-DHBA in hippocampus and caudate; Figure 33, DOPAC in piriform, parietal and frontal cortices; Figure 34, DOPAC in hippocampus and

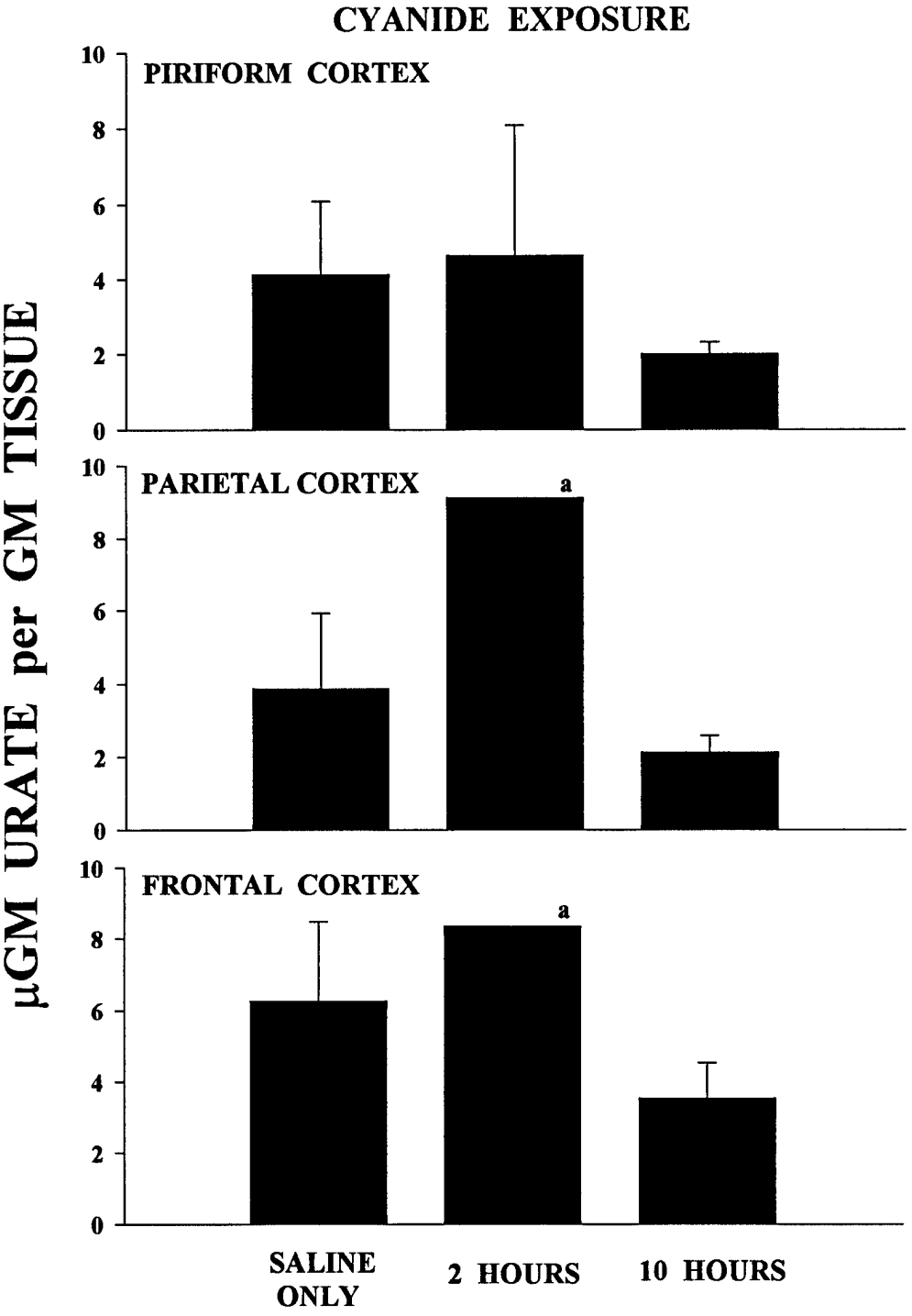

Figure 11. Concentrations of urate (μg/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized as in methods for analyses (N=1 to 3). a = only one result available. No significant differences between groups were found.

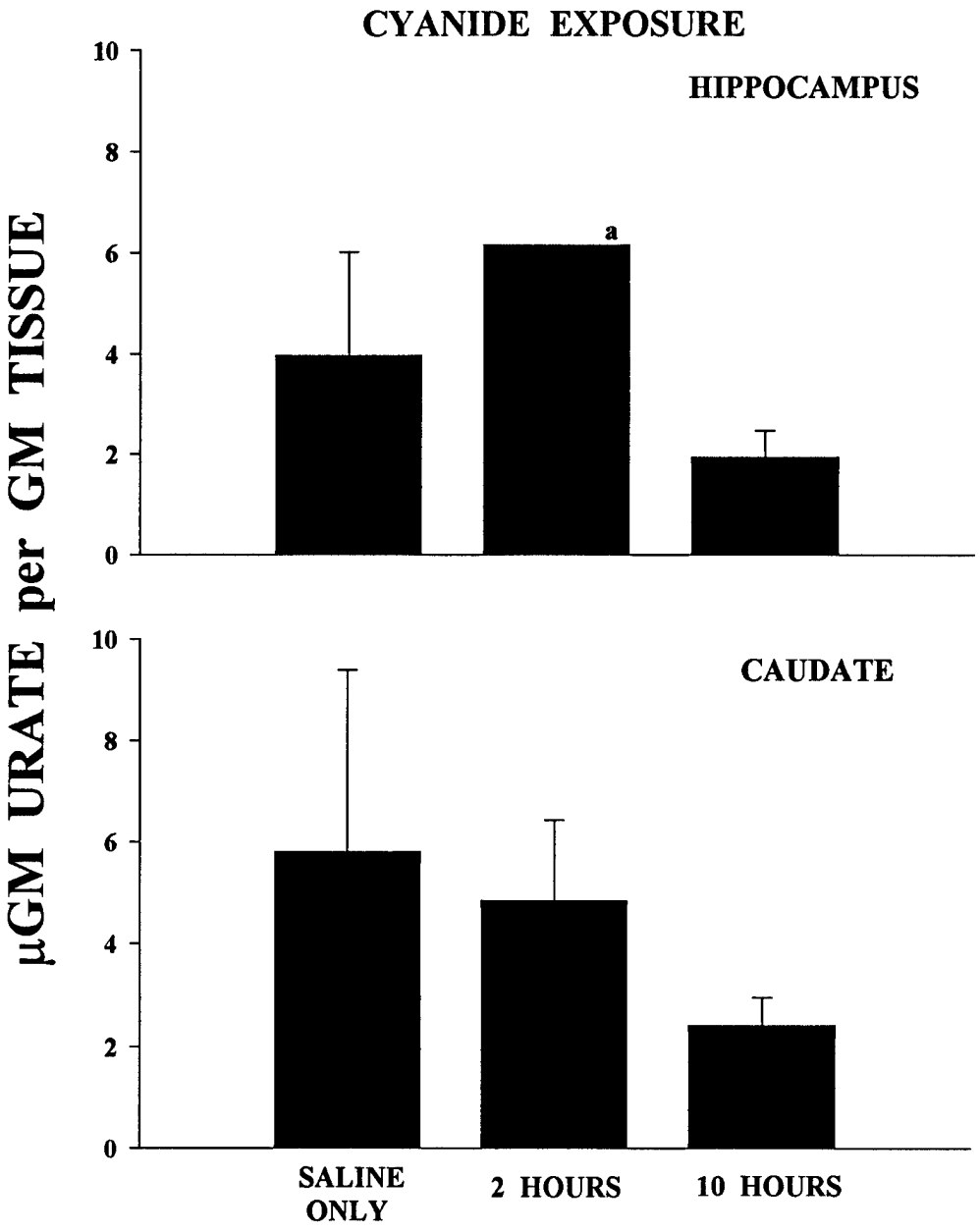

Figure 12. Concentrations of urate (μg/g tissue) in tissue homogenates of hippocampus and caudate after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=1 to 3). a = only one result available. No significant differences between groups were found.

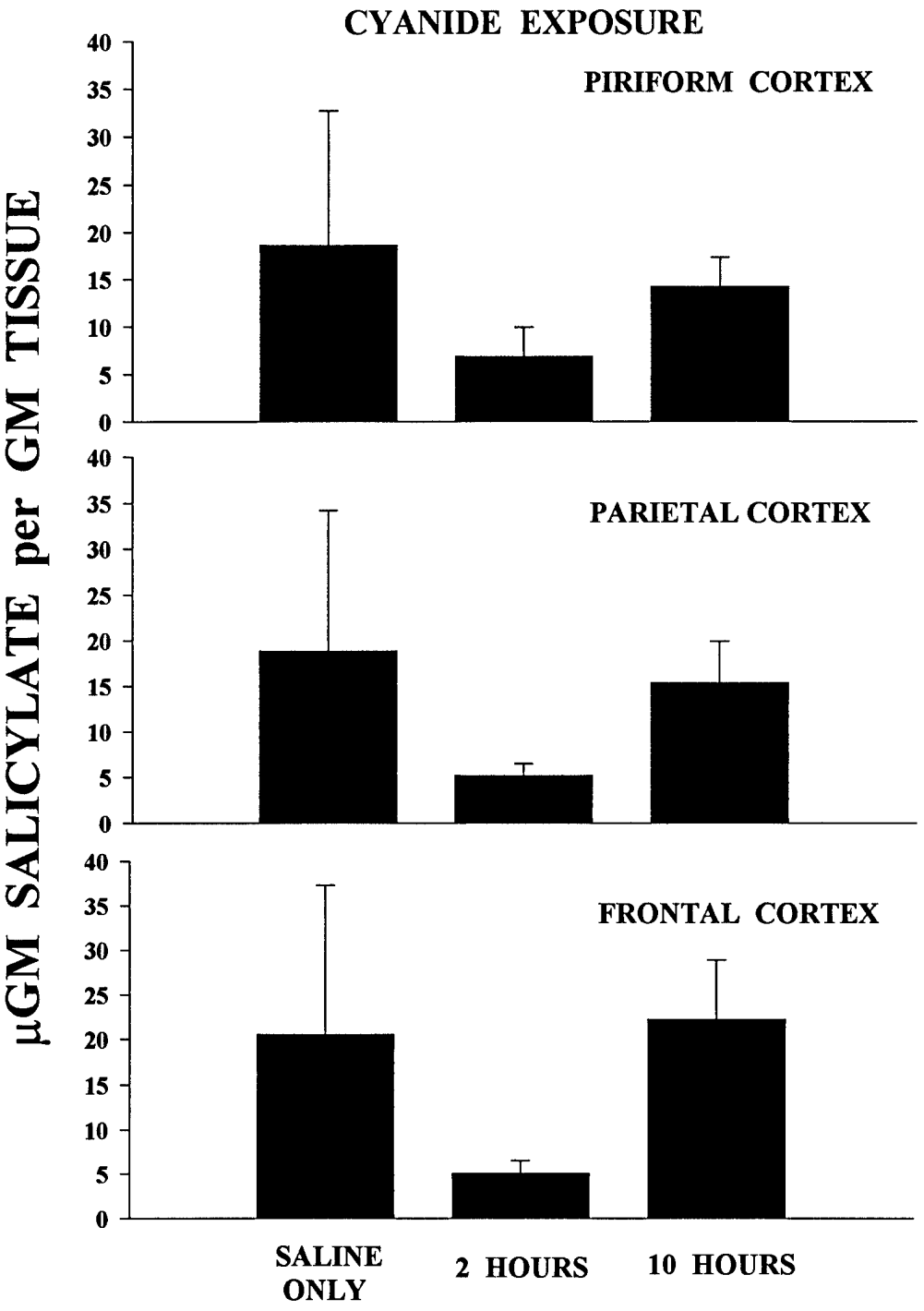

Figure 13. Concentrations of salicylate (μg/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized as in methods for analyses (N=2 to 4). No significant differences between groups were found.

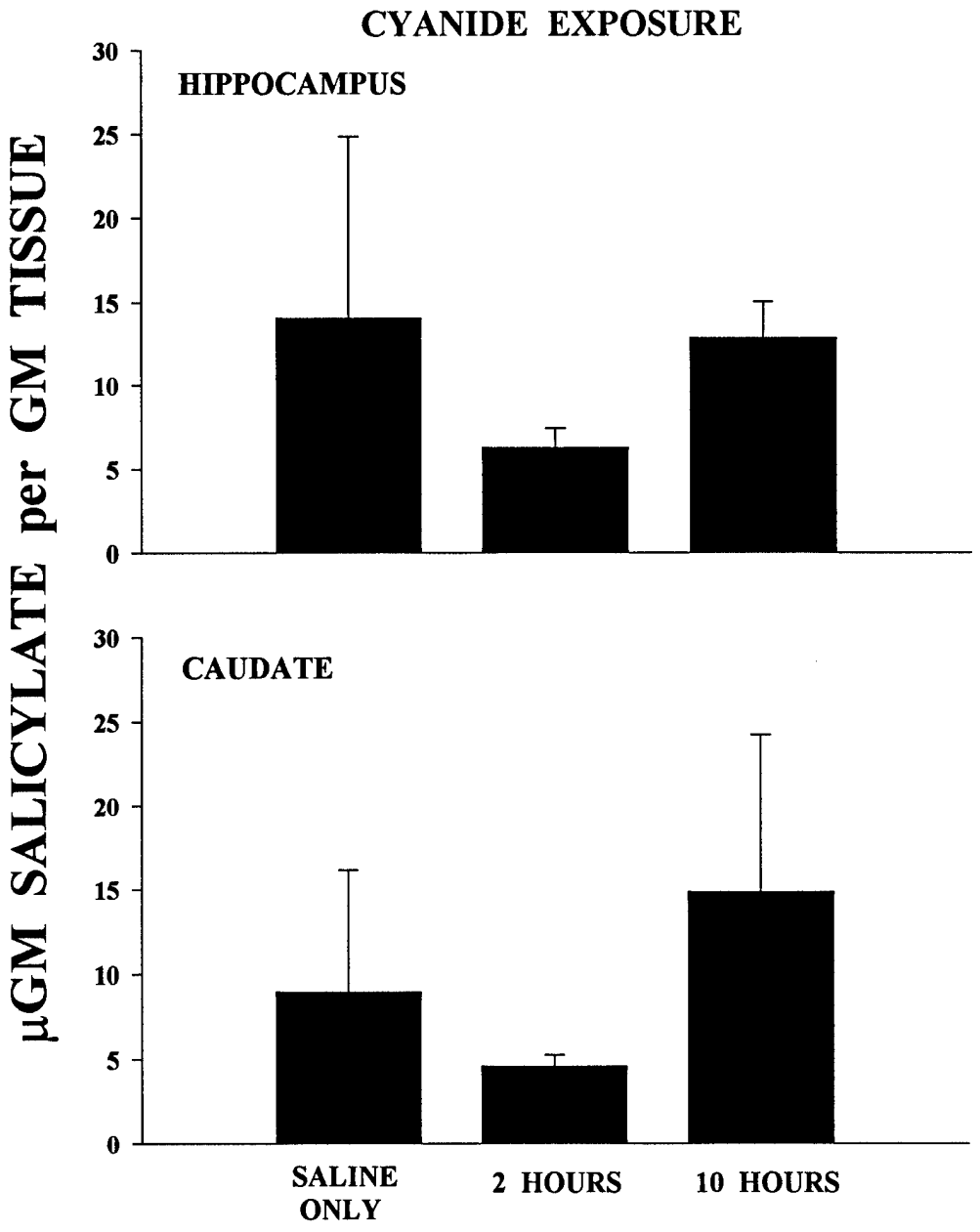

Figure 14. Concentrations of salicylate (µg/g tissue) in tissue homogenates of hippocampus and caudate after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=2 to 4). No significant differences between groups were found.

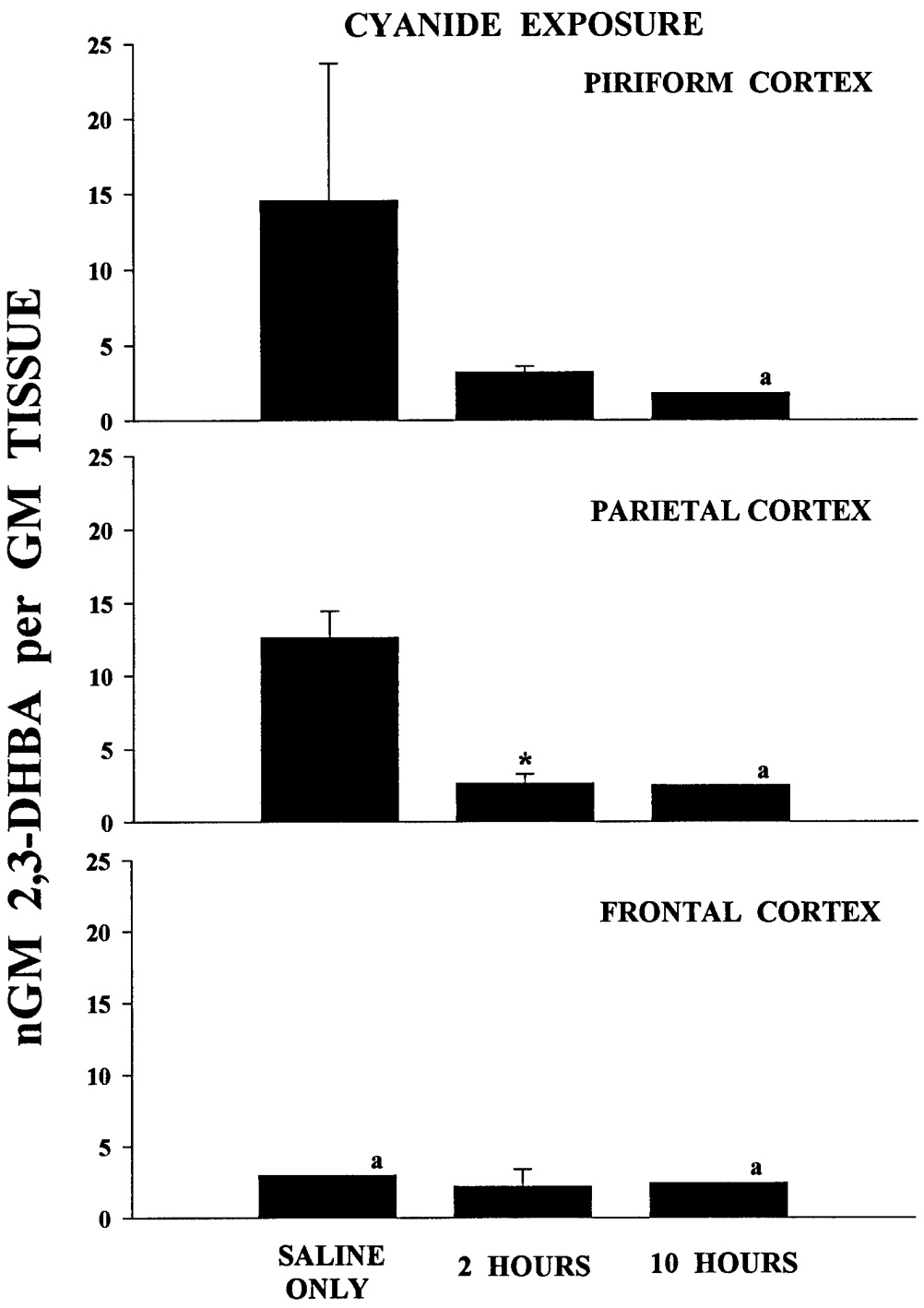

Figure 15. Concentrations of 2,3-DHBA (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized as in methods for analyses (N=2 to 3). a = detected in only one rat. * = significantly different from SALINE ONLY at $P<0.05$ using one-way ANOVA and Dunnett's post-hoc test.

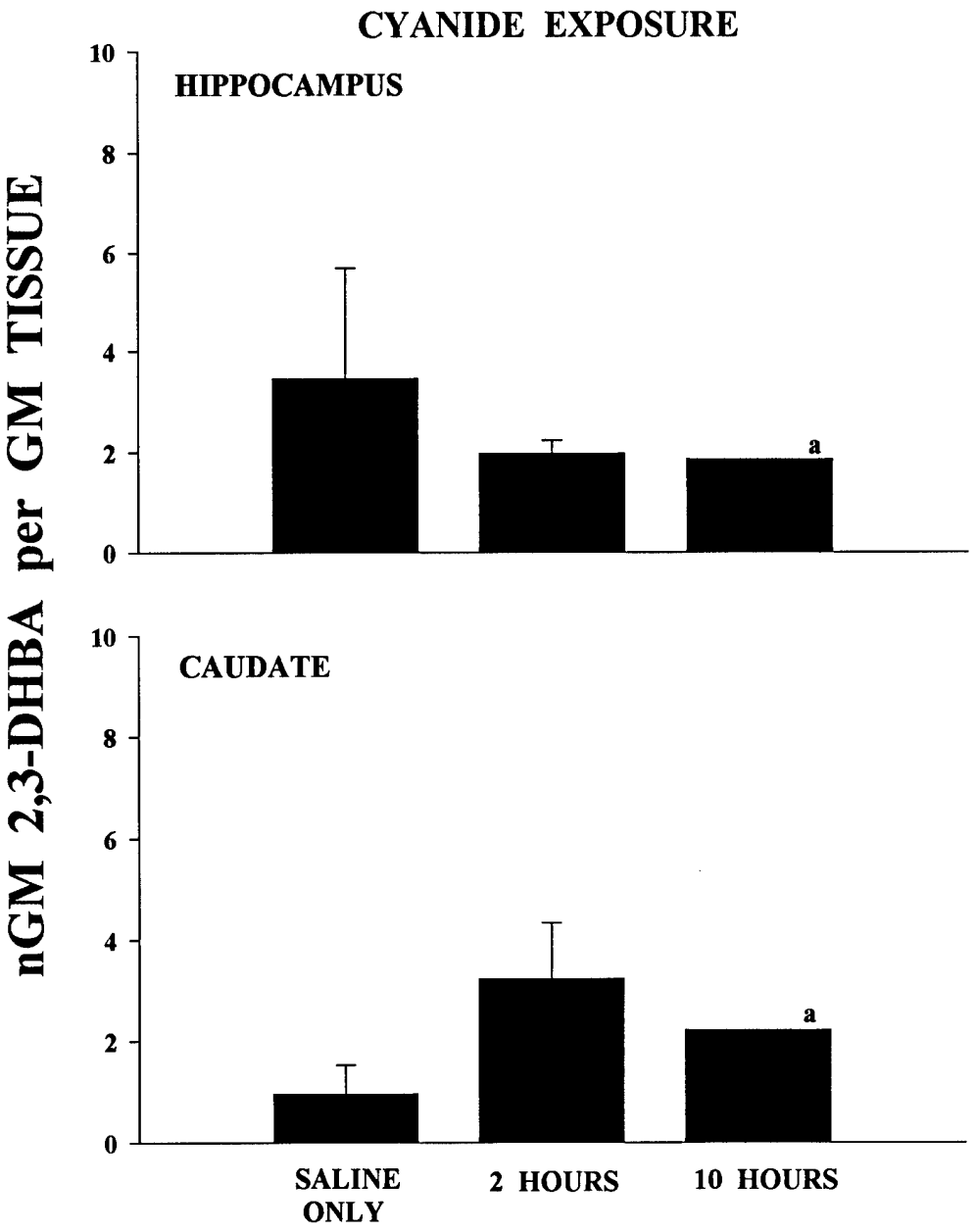

Figure 16. Concentrations of 2,3-DHBA (ng/g tissue) in tissue homogenates of hippocampus and caudate after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=2 to 4). a = detected in only one rat. No significant differences between groups were found.

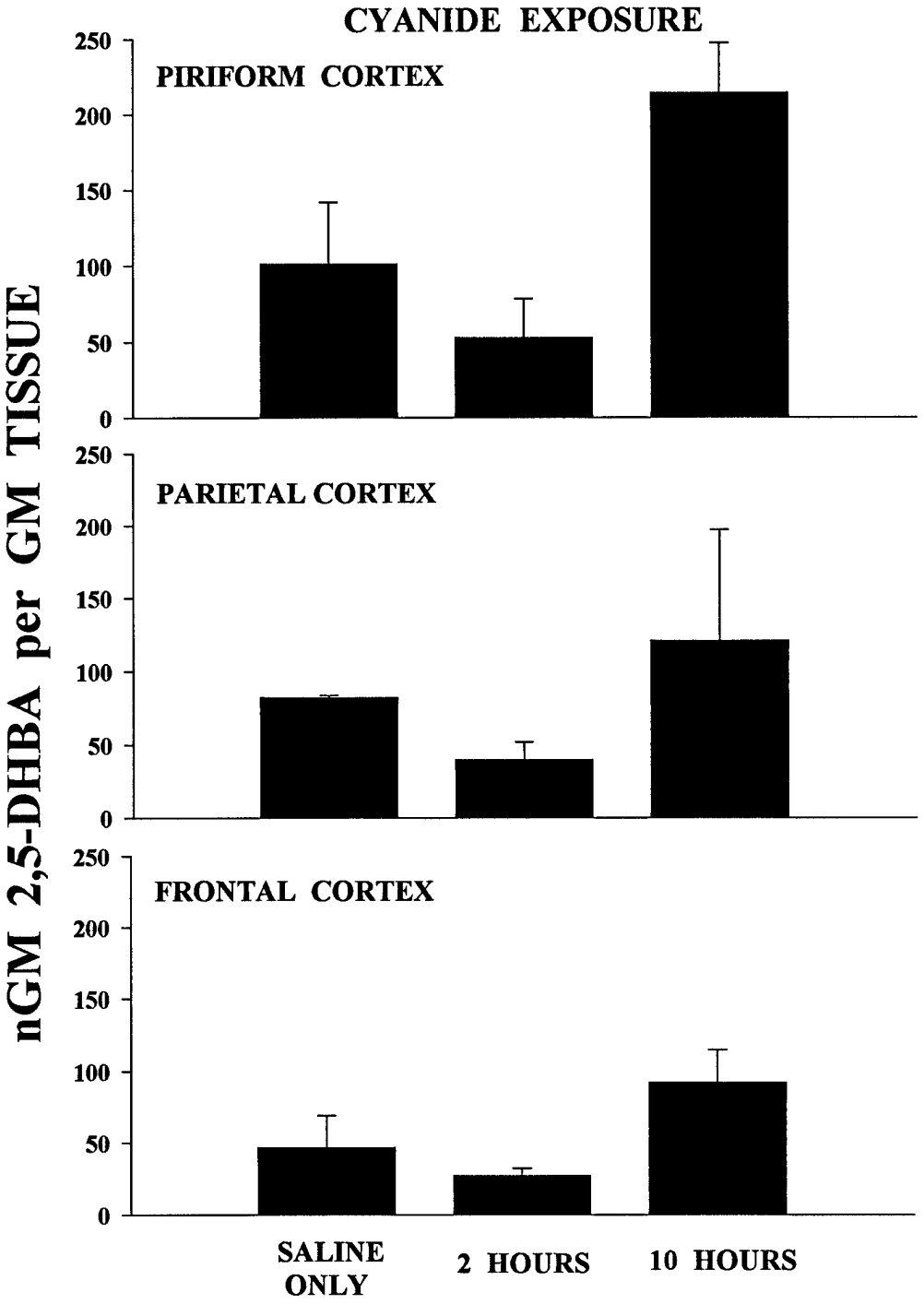

Figure 17. Concentrations of 2,5-DHBA (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized as in methods for analyses (N=2 to 4). No significant differences between groups were found.

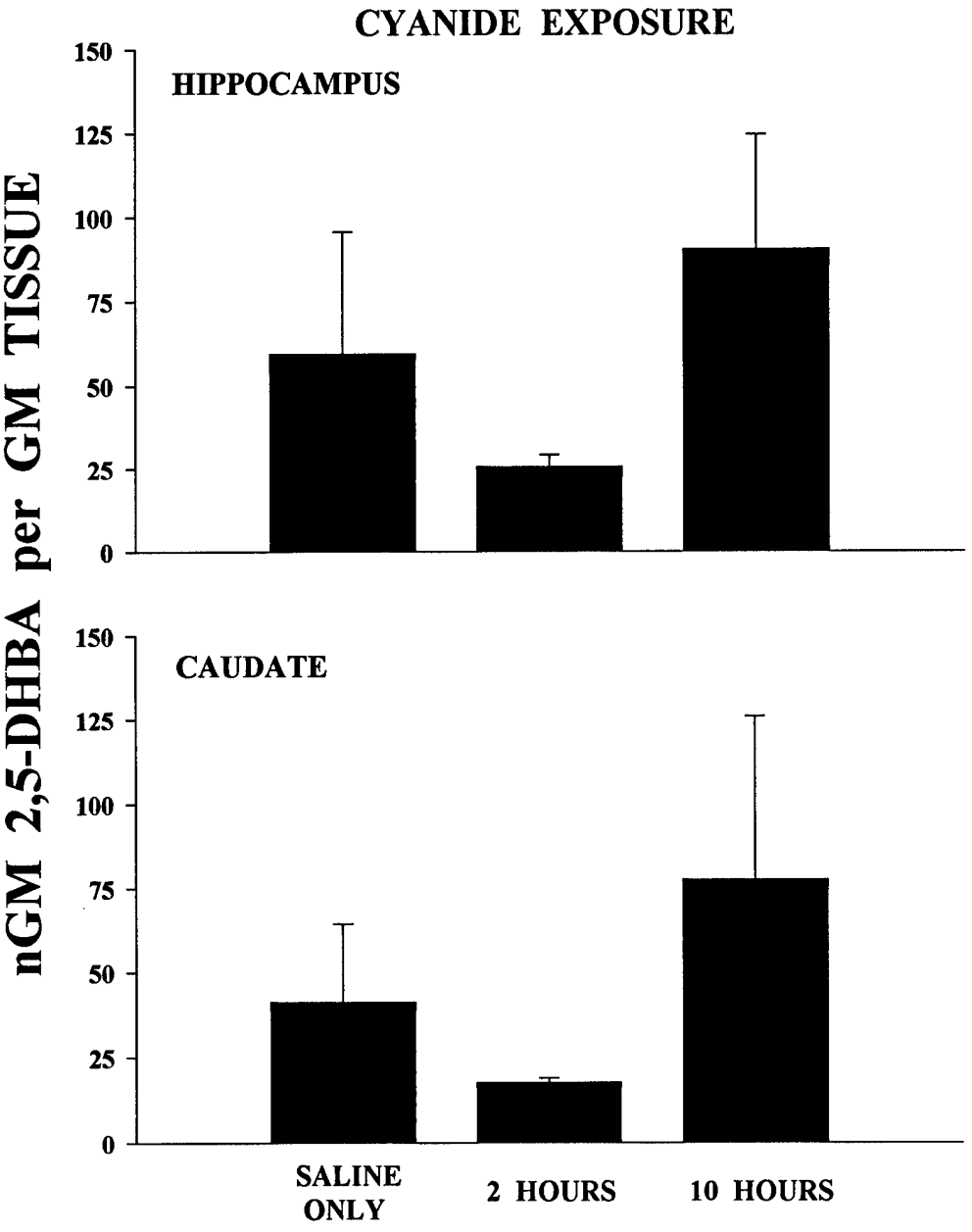

Figure 18. Concentrations of 2,5-DHBA (ng/g tissue) in tissue homogenates of hippocampus and caudate after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=2 to 4). No significant differences between groups were found.

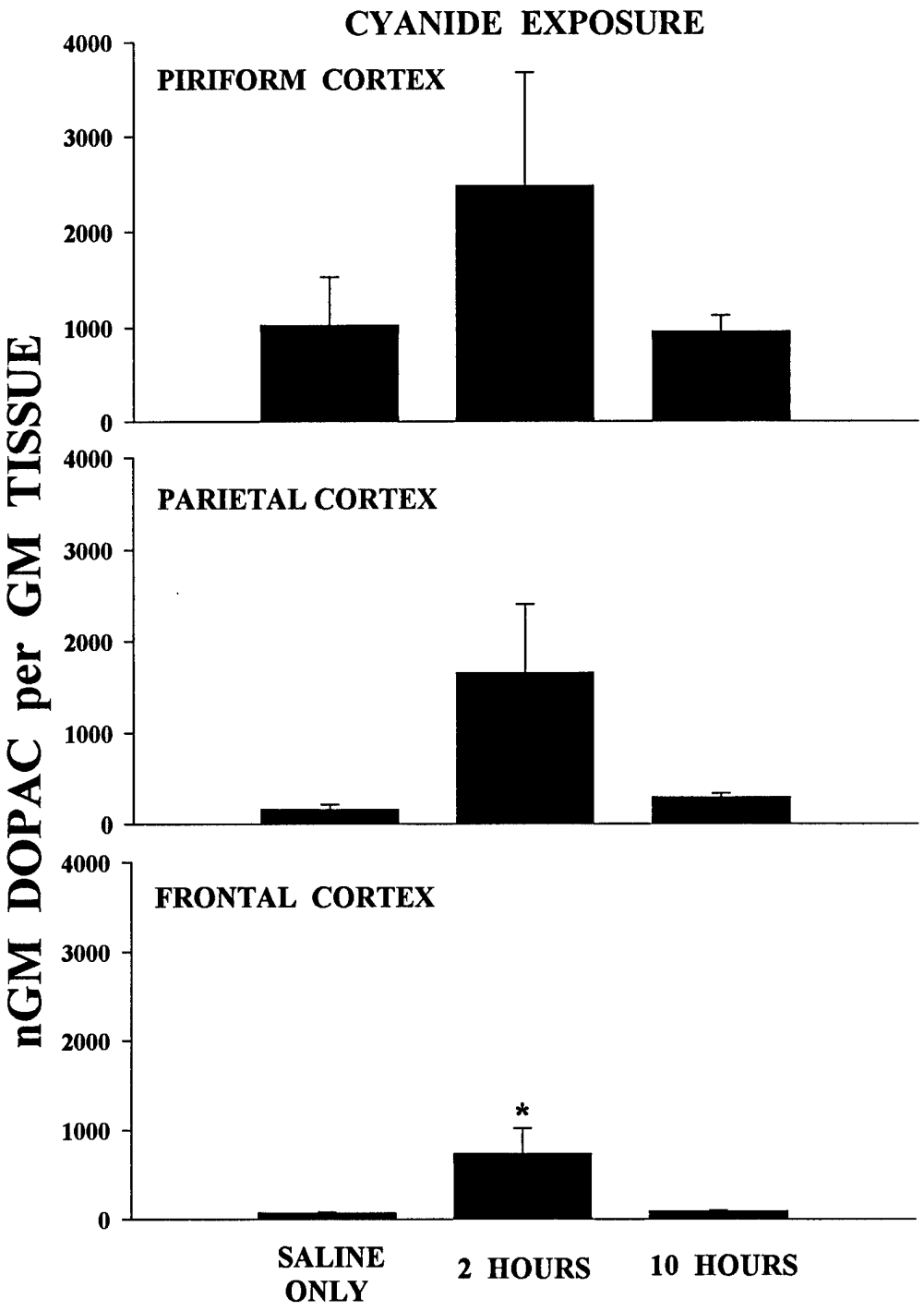

Figure 19. Concentrations of DOPAC (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=2 to 4). * = significantly different form SALINE ONLY at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

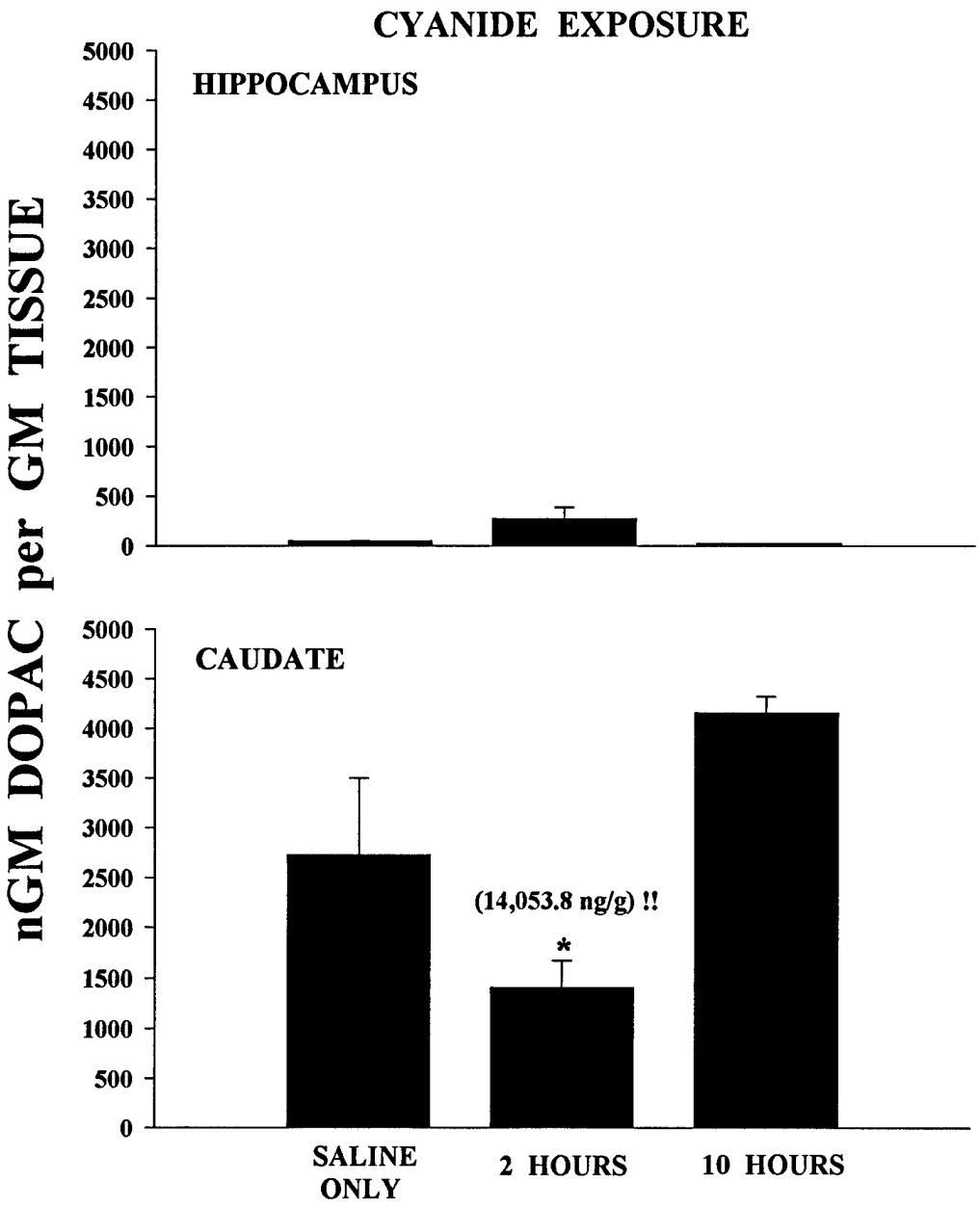

Figure 20. Concentrations of DOPAC (ng/g tissue) in tissue homogenates of hippocampus and caudate after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=2 to 4).
*** = significantly different from SALINE ONLY at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.**
!! = bar shown at 1/10 actual value to better visualize other data. (actual value given in parentheses.)

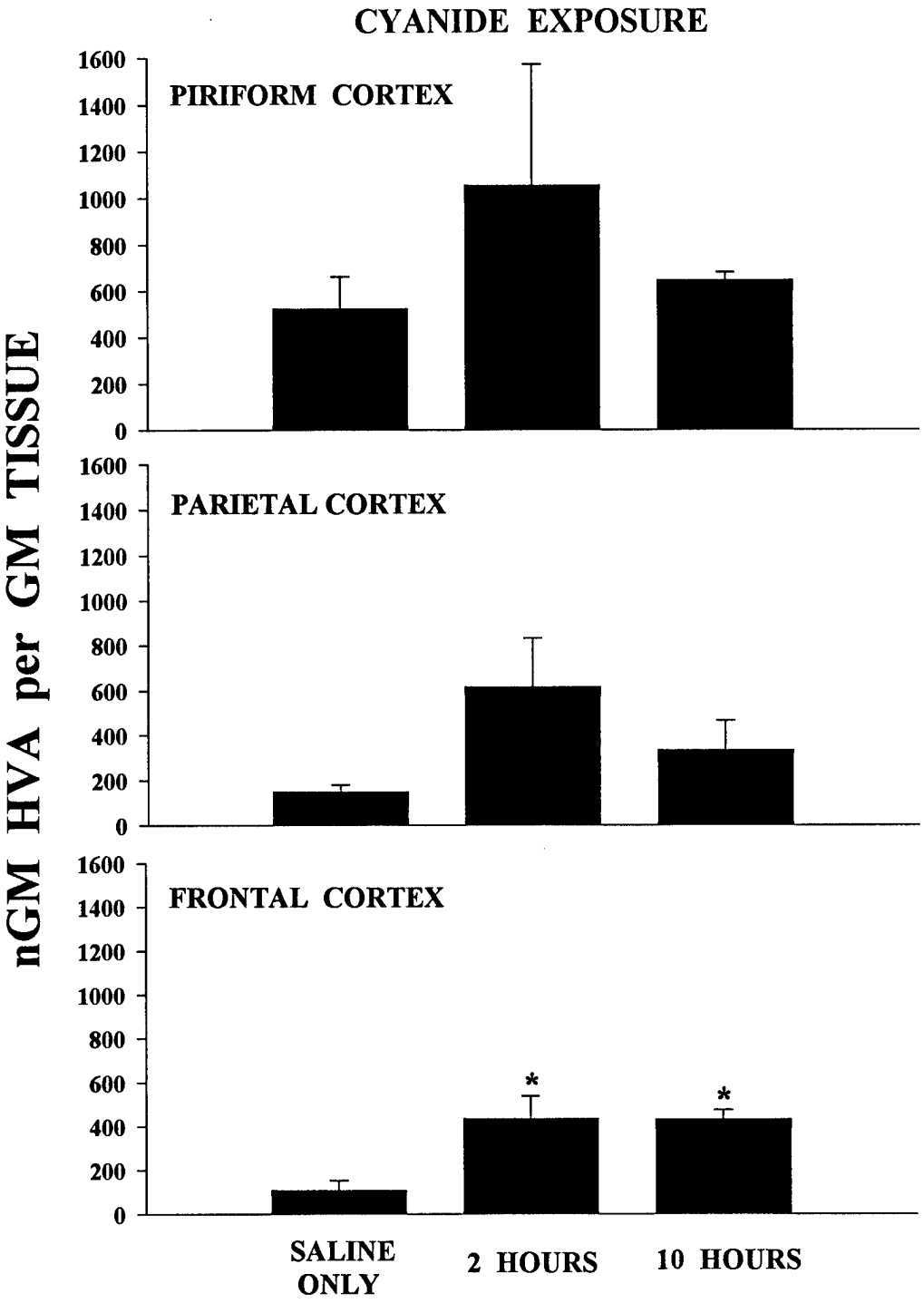

Figure 21. Concentrations of HVA (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized as in methods for analyses (N=2 to 4). * = significantly different from SALINE ONLY at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

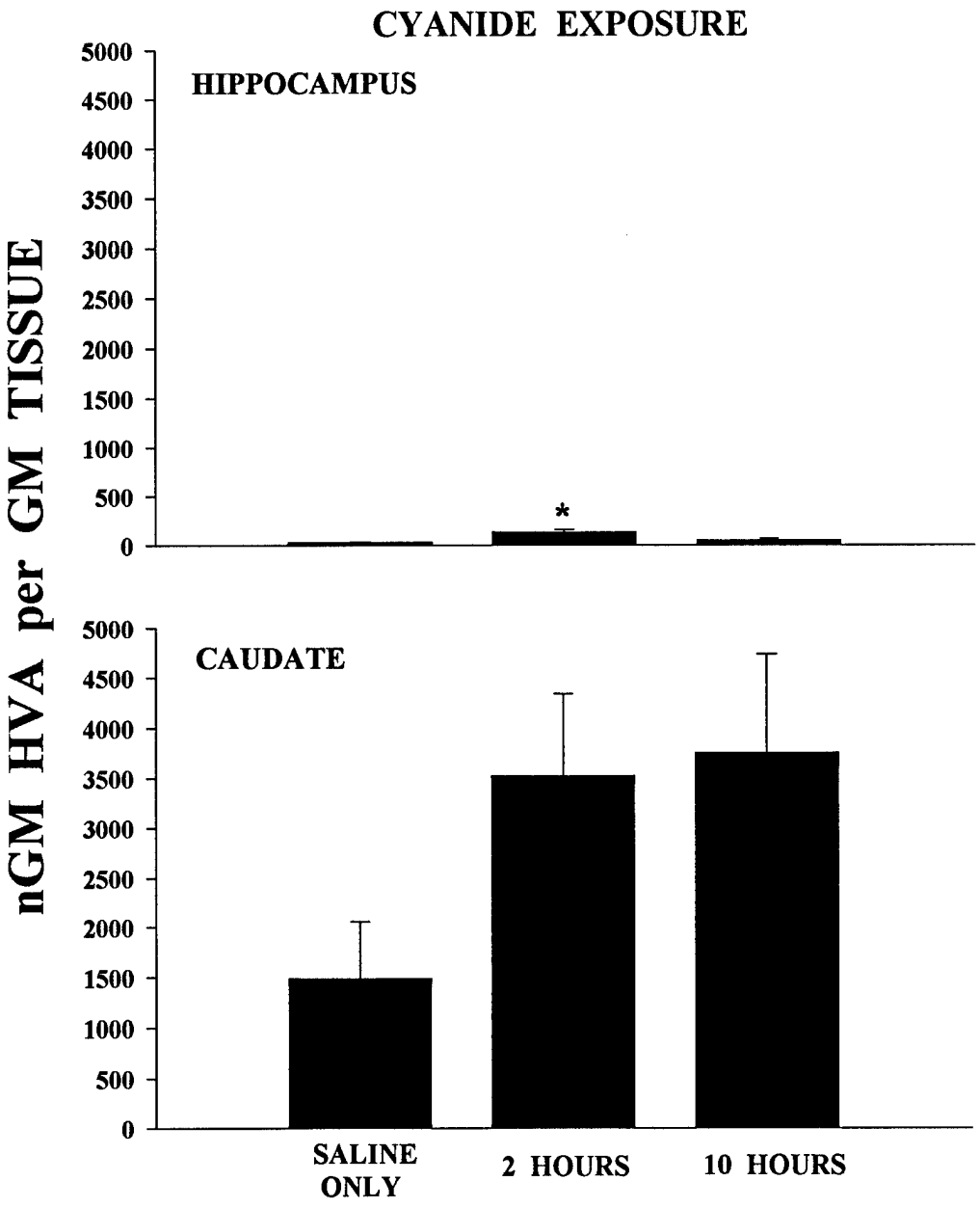

Figure 22. Concentrations of HVA (ng/g tissue) in tissue homogenates of hippocampus and caudate after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=2 to 4). * = significantly different from SALINE ONLY at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

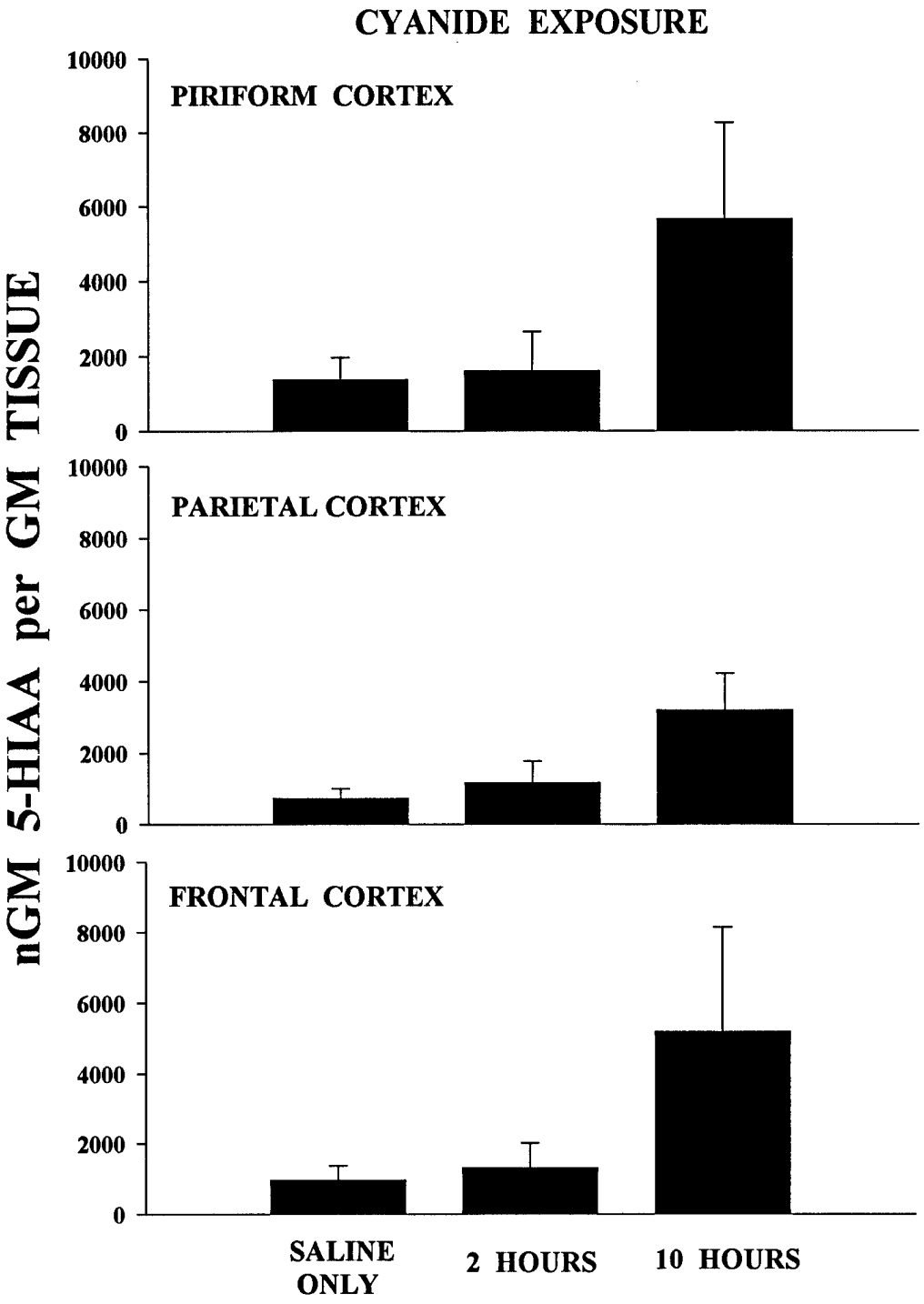

Figure 23. Concentrations of 5-HIAA (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=2 to 4). No significant differences between groups were found.

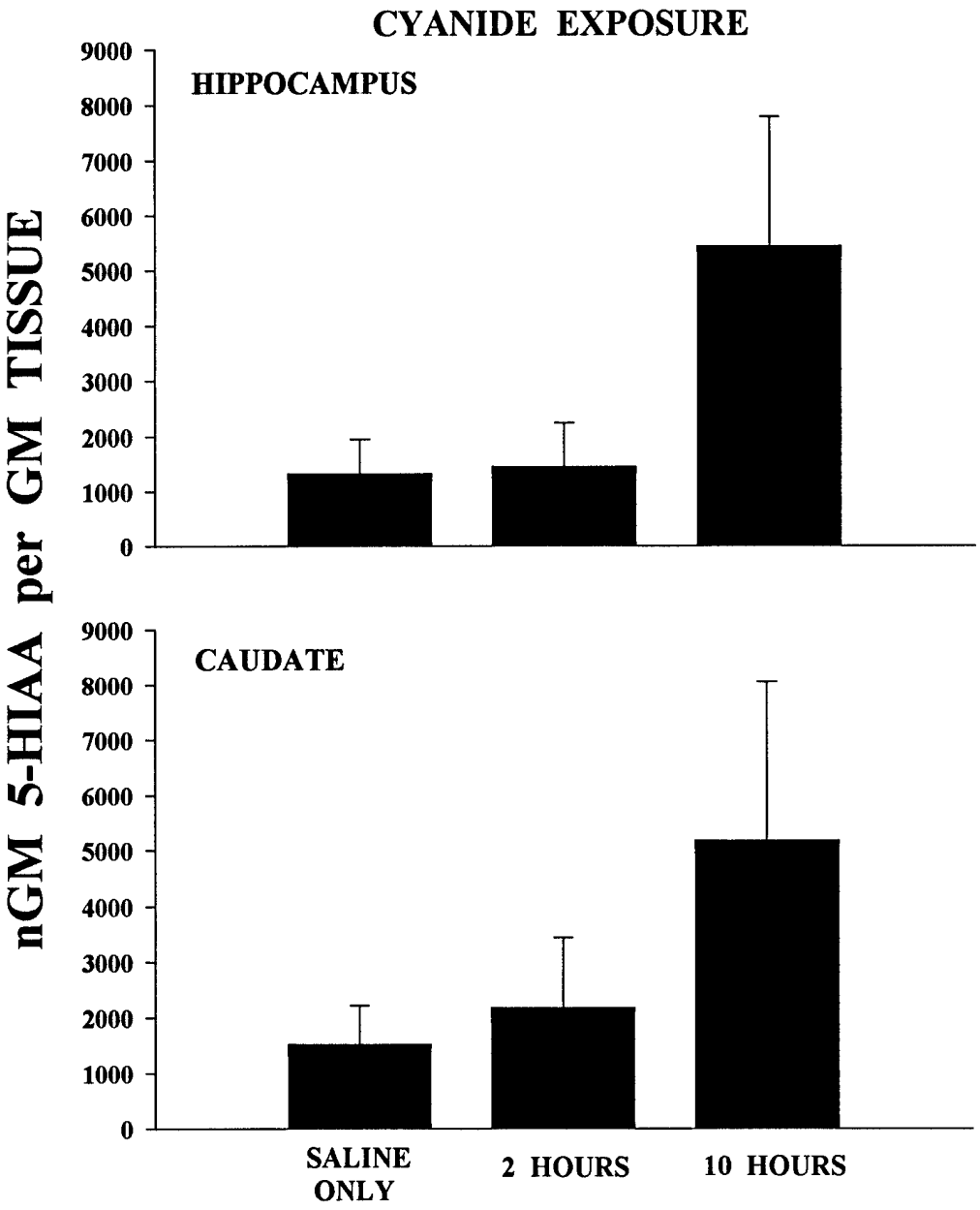

Figure 24. Concentrations of 5-HIAA (ng/g tissue) in tissue homogenates of hippocampus and caudate after cyanide exposure (0.1 mg/min. intermittently during 90 minutes; iv infusion). Rats were without righting reflex during this time. Sacrifice times shown are from onset of infusion. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected and immediately homogenized and analyzed as in methods (N=2 to 4). No significant differences between groups were found.

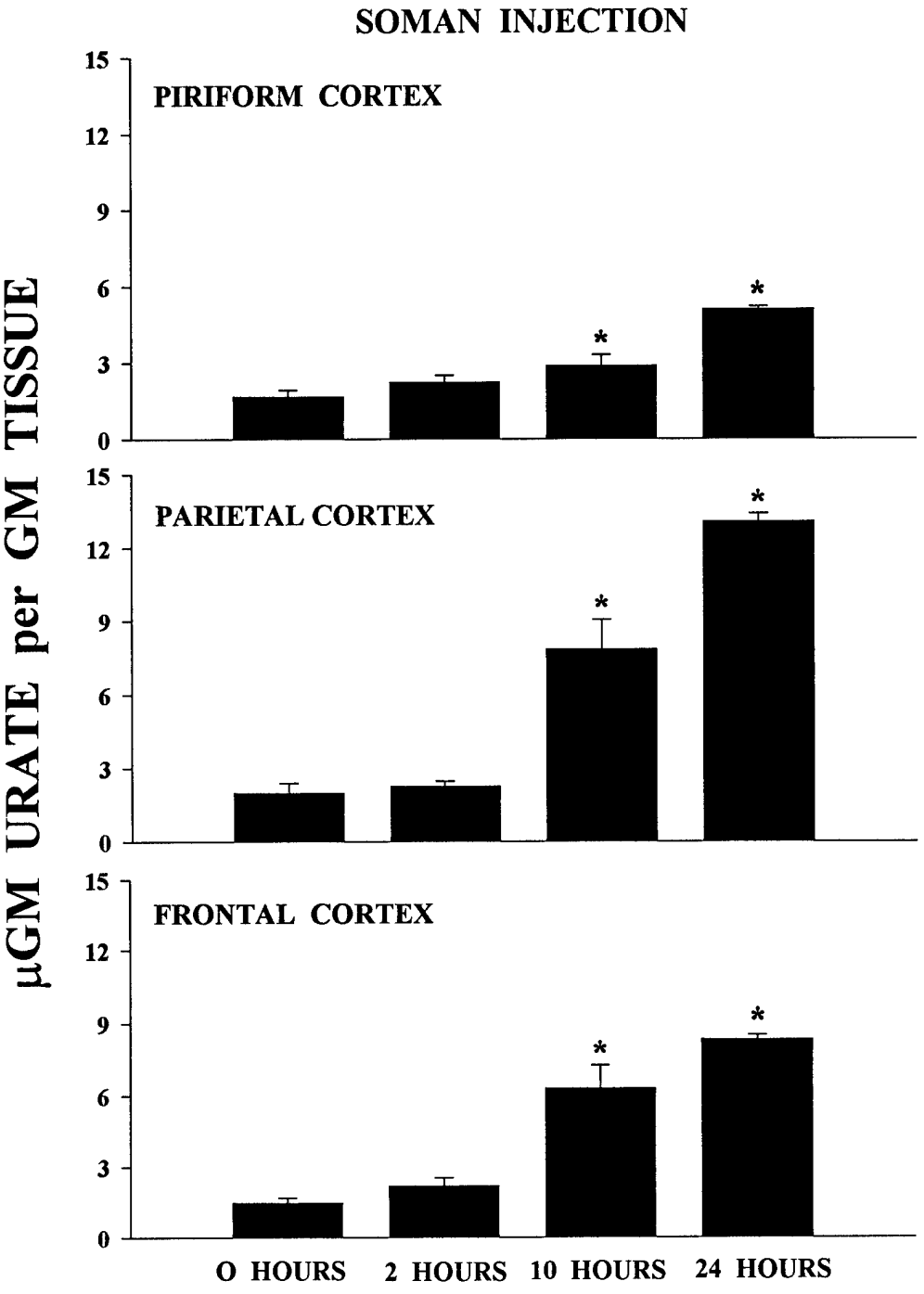

Figure 25. Concentrations of urate (μg/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after an injection of soman (80-90 μg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses (N=2 to 10). * = significantly different from 0 HOURS at $P<0.05$ using one-way ANOVA and Dunnett's post-hoc test.

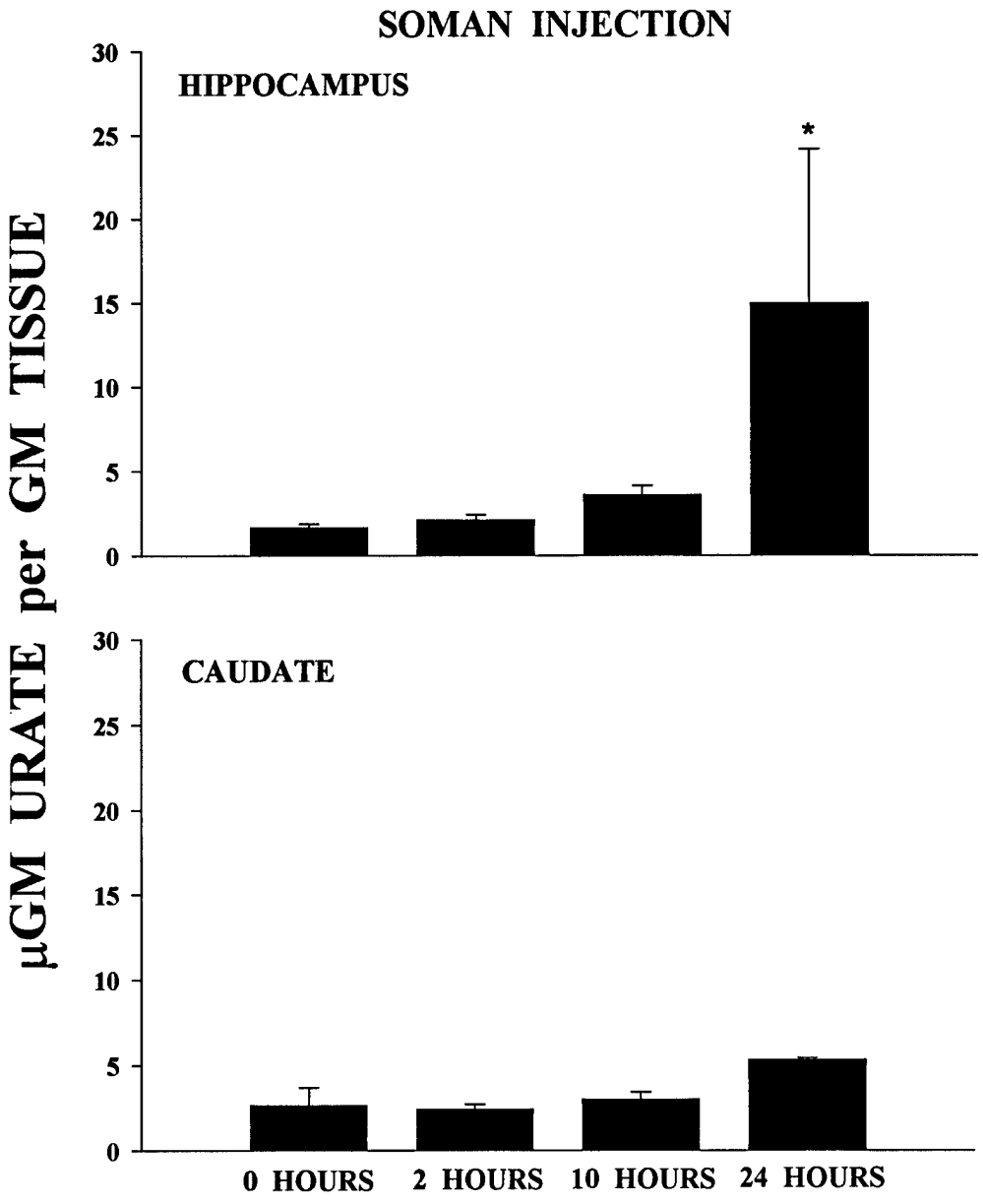

Figure 26. Concentrations of urate (μg/g tissue) in tissue homogenates of hippocampus and caudate after an injection of soman (80-90 mg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized and analyzed as in methods (N= 2 to 10). * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

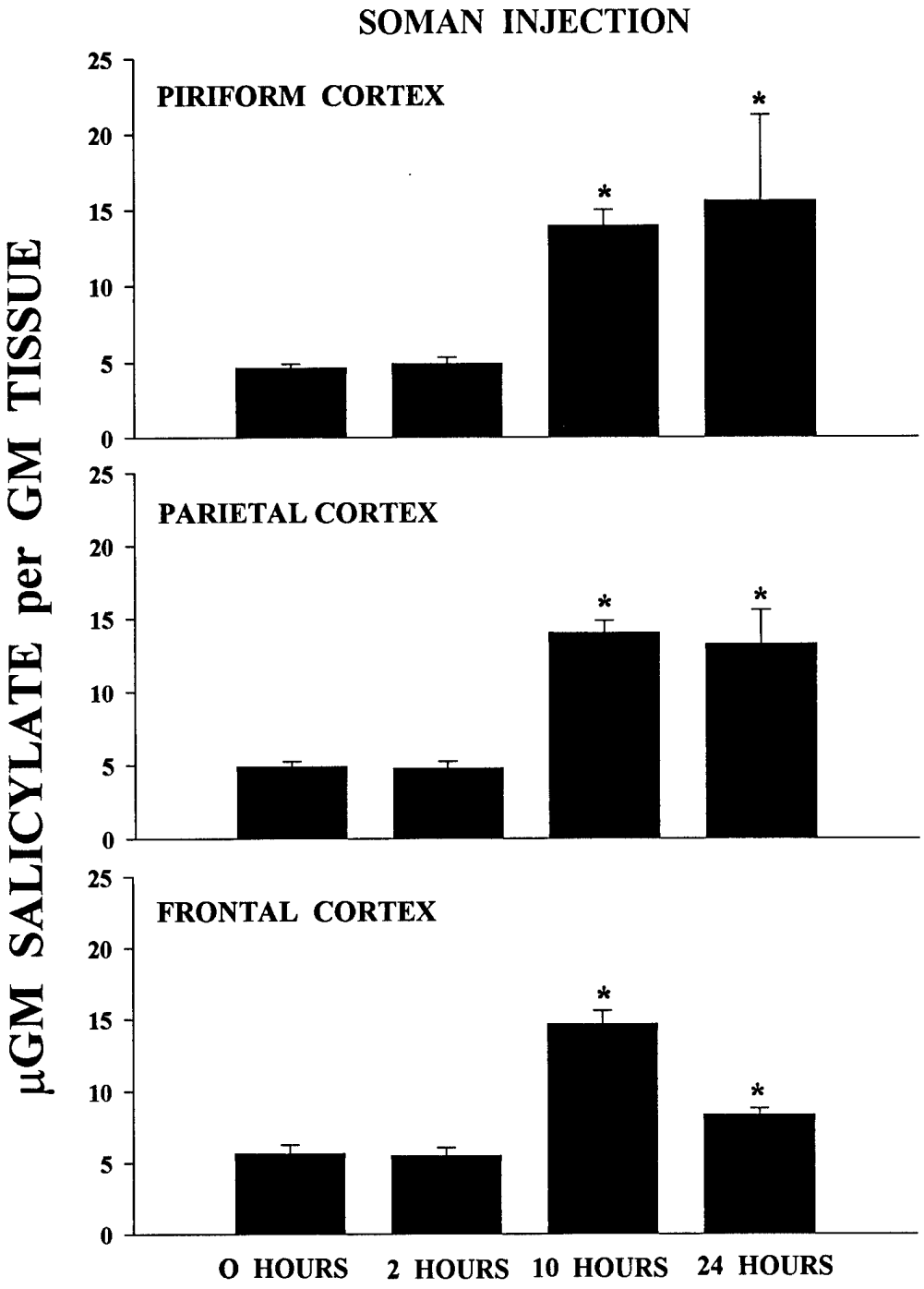

Figure 27. Concentrations of salicylate (μg/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after an injection of soman (80-90 μg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses (N=2 to 10). * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

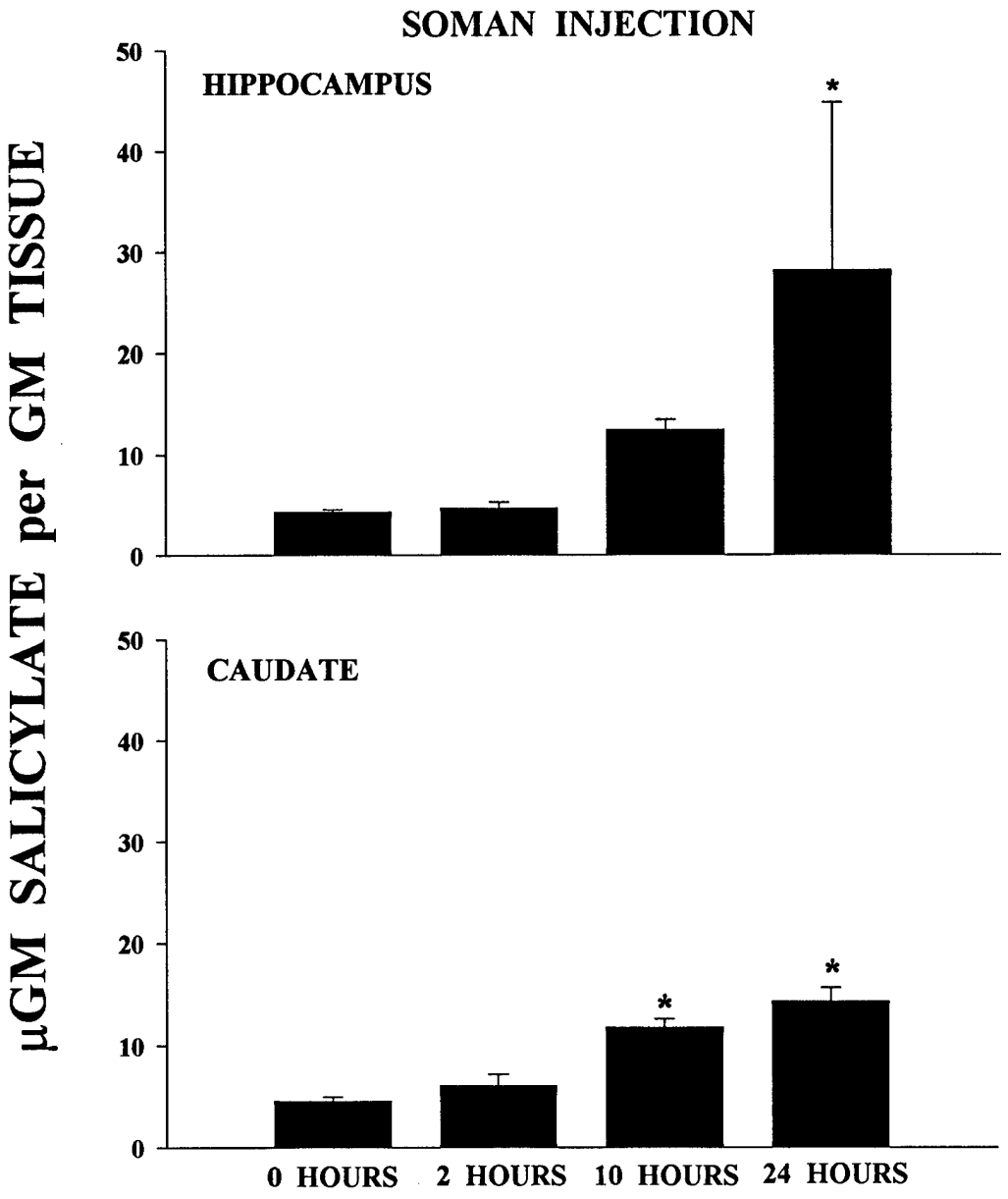

Figure 28. Concentrations of salicylate (µg/g tissue) in tissue homogenates of hippocampus and caudate after an injection of soman (80-90 mg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized Aand analyzed as in methods (N= 2 to 10). * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

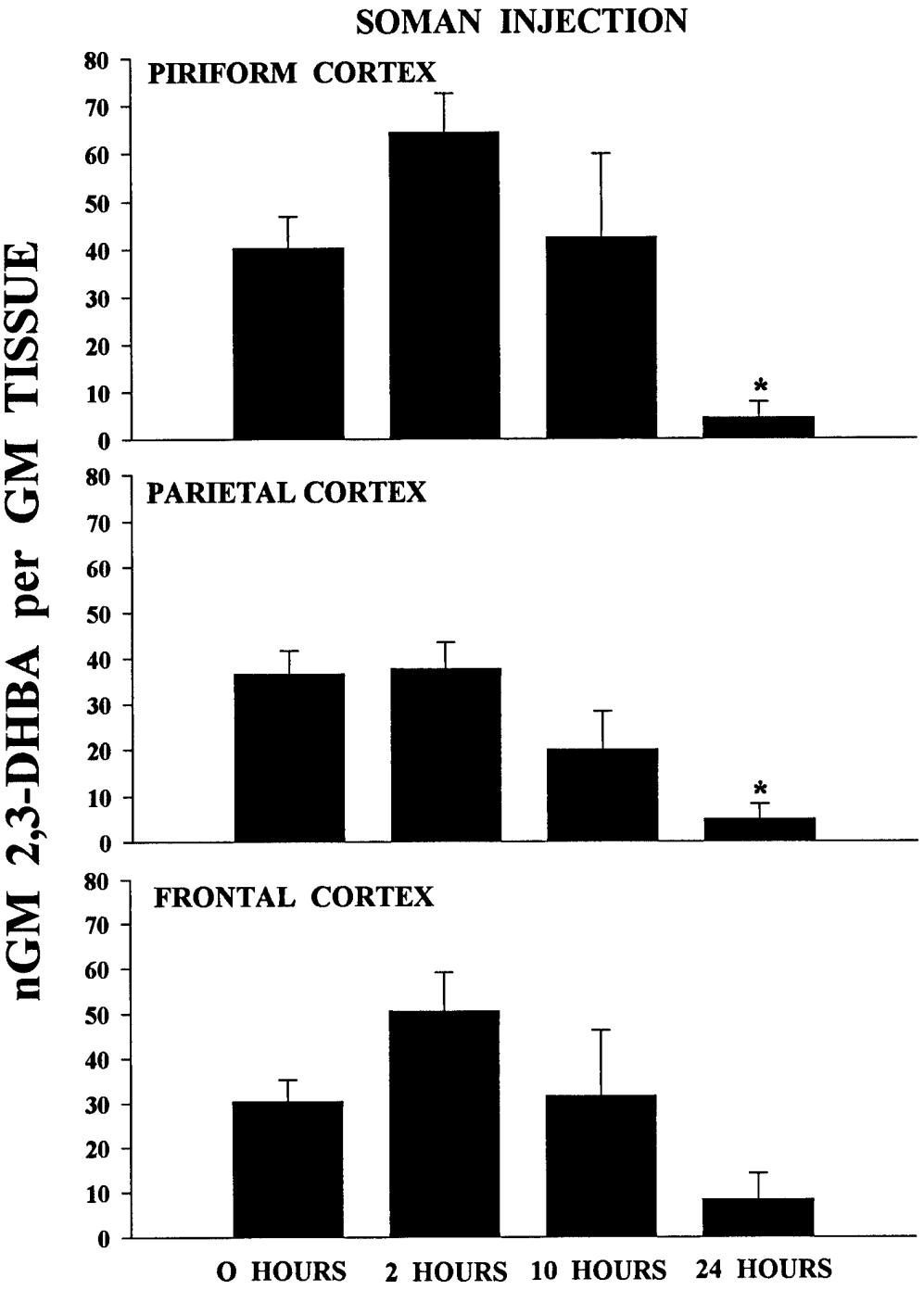

Figure 29. Concentrations of 2,3-DHBA (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after an injection of soman (80-90 μg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses (N=2 to 10). * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

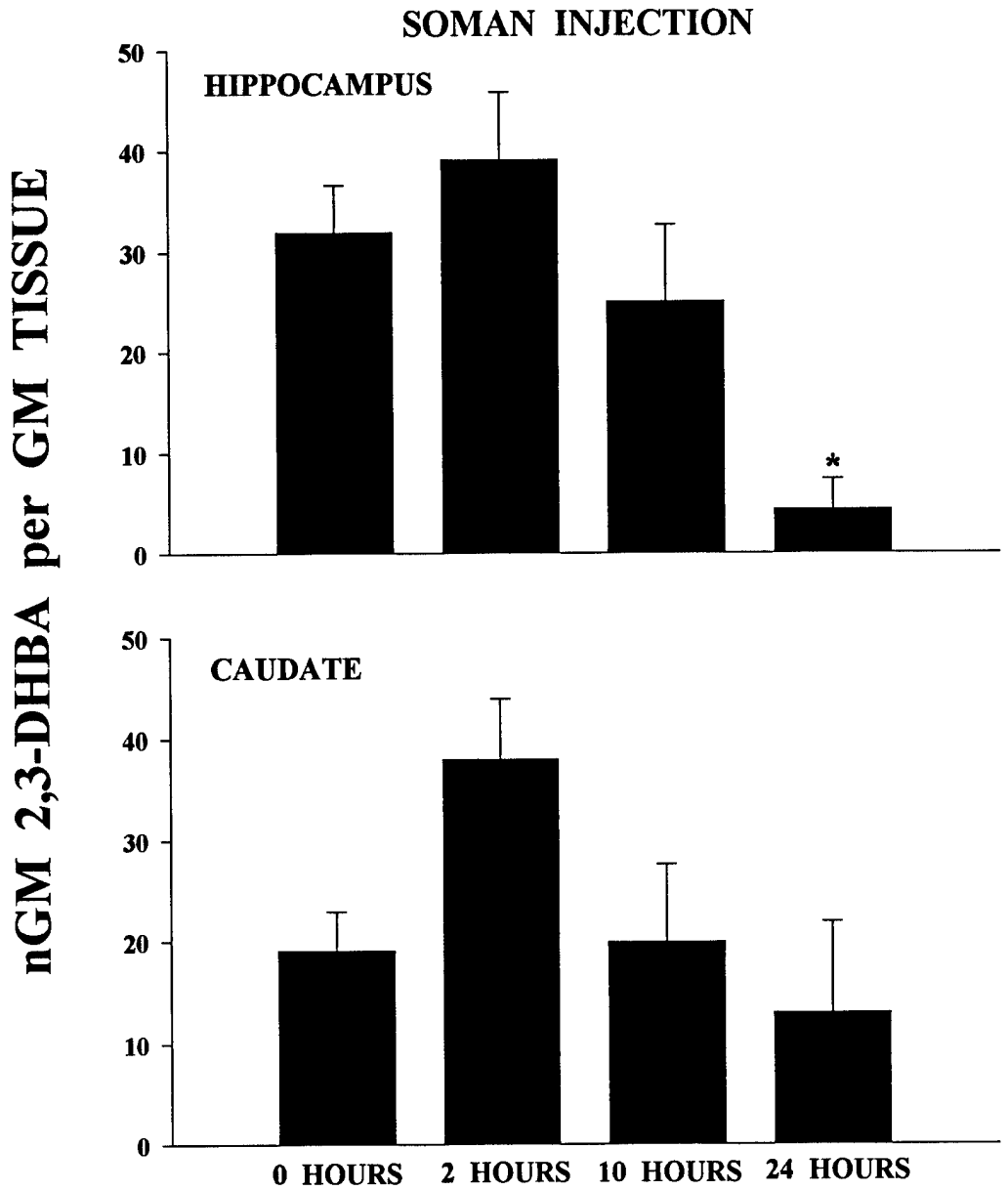

Figure 30. Concentrations of 2,3-DHBA (ng/g tissue) in tissue homogenates of hippocampus and caudate after an injection of soman (80-90 mg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses. (N= 3 to 9) * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

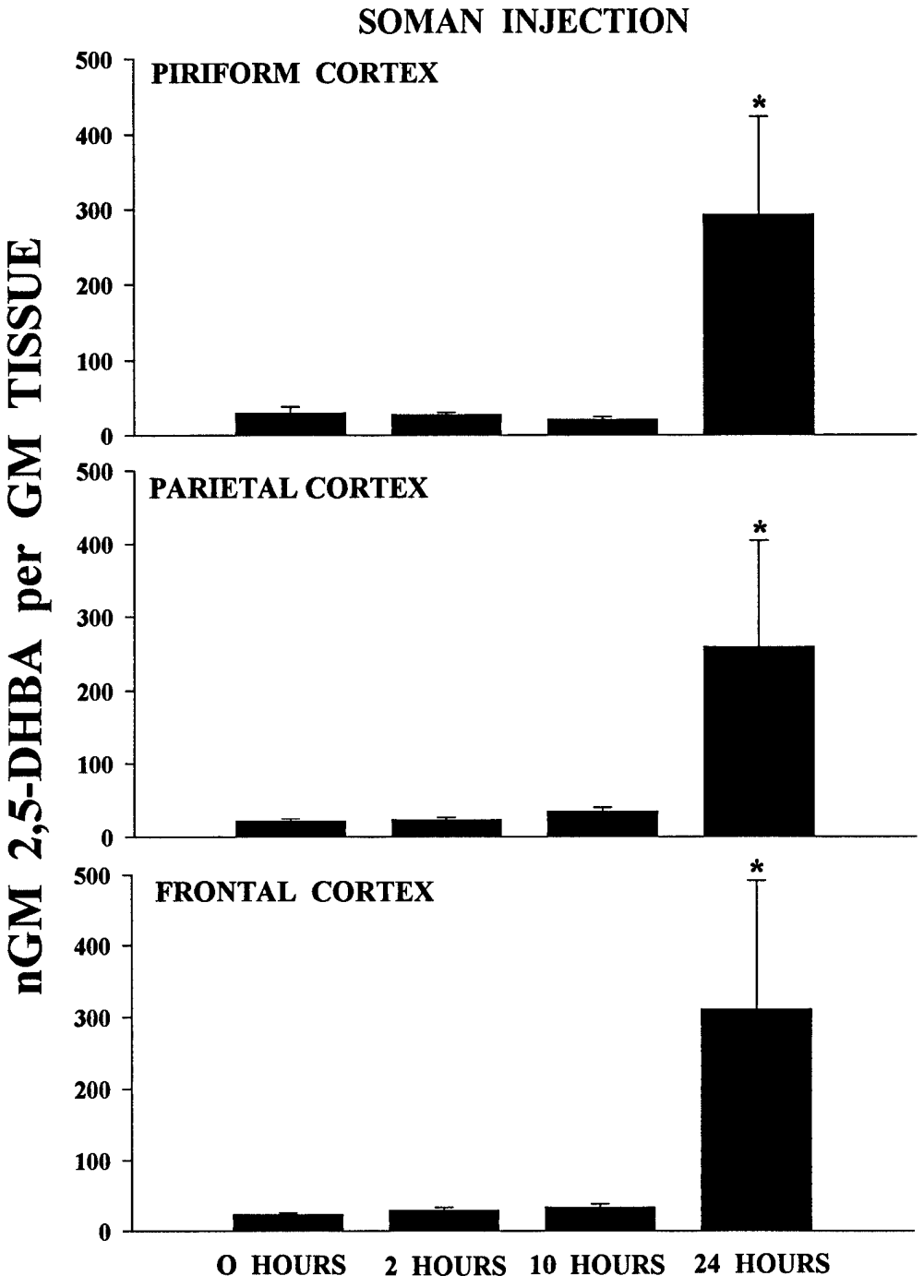

Figure 31. Concentrations of 2,5-DHBA (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after an injection of soman (80-90 μg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses (N=3 to 6). * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

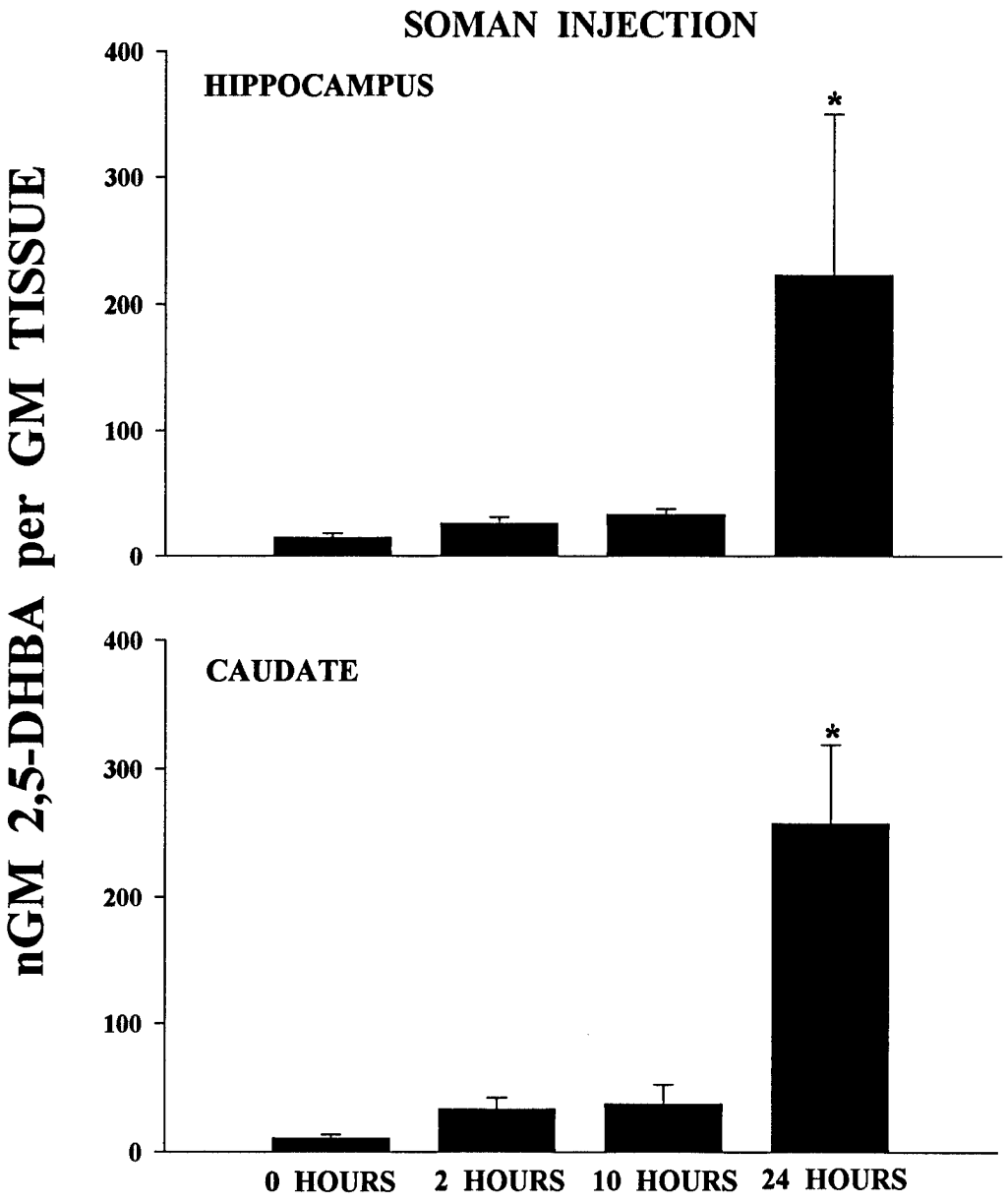

Figure 32. Concentrations of 2,5-DHBA (ng/g tissue) in tissue homogenates of hippocampus and caudate after an injection of soman (80-90 mg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses. (N= 3 to 7) * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

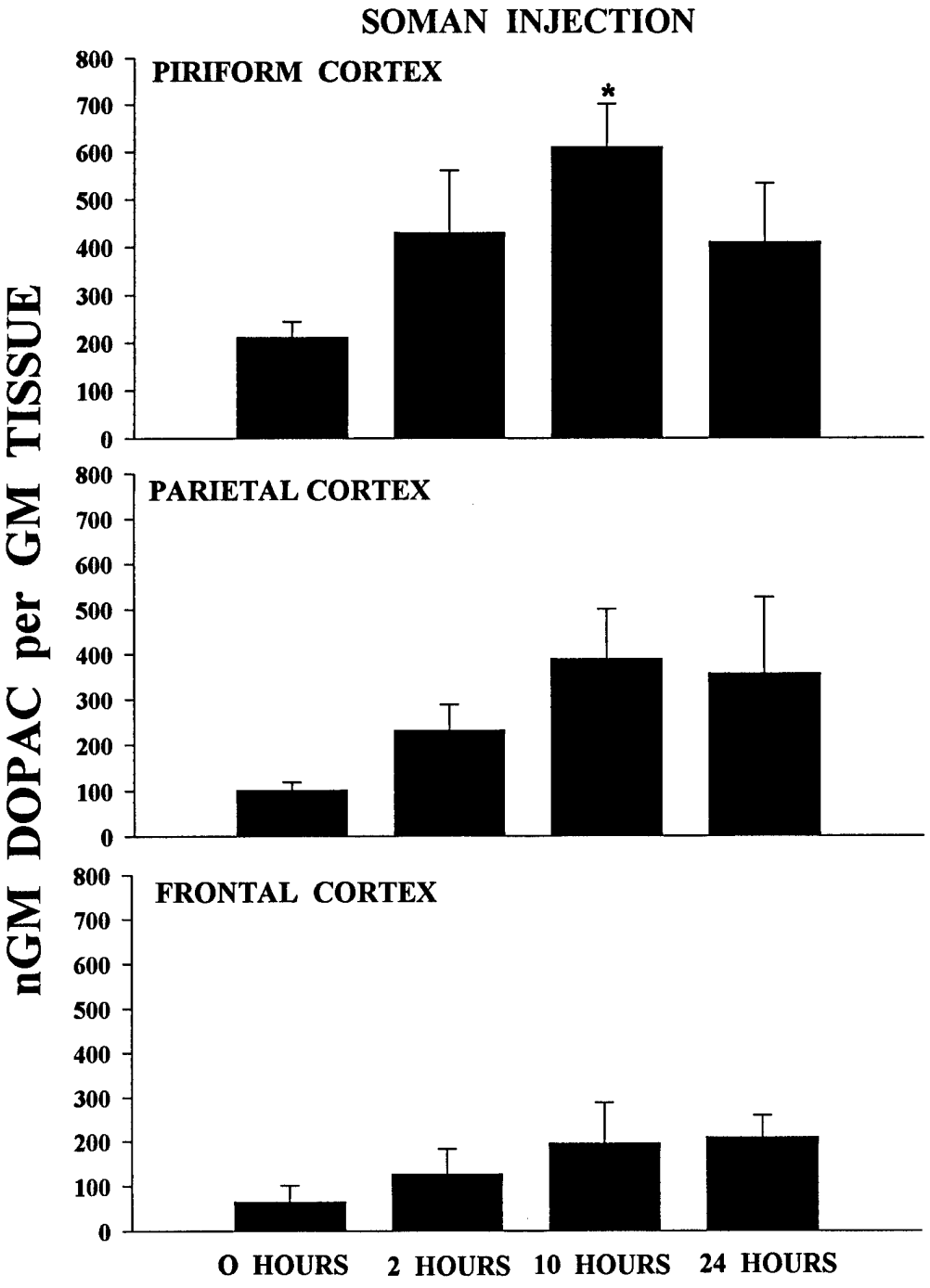

Figure 33. Concentrations of DOPAC (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after an injection of soman (80-90 μg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses (N=2 to 9). * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

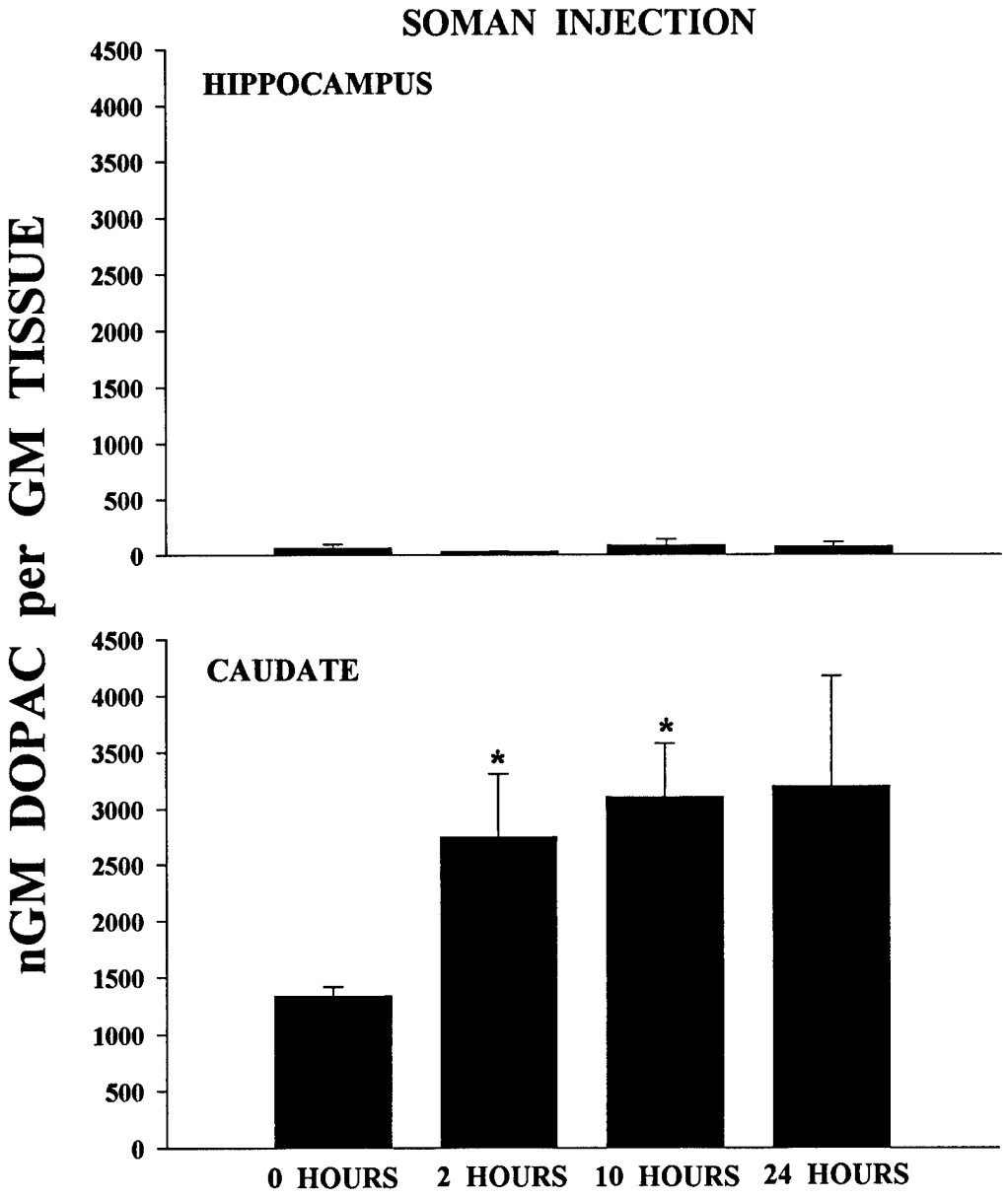

Figure 34. Concentrations of DOPAC (ng/g tissue) in tissue homogenates of hippocampus and caudate after an injection of soman (80-90 mg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses. (N= 2 to 9) * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

caudate; Figure 35, HVA in piriform, parietal and frontal cortices; Figure 36, HVA in hippocampus and caudate; Figure 37, 5-HIAA in piriform, parietal and frontal cortices; Figure 38, 5-HIAA in hippocampus and caudate. Increases in urate, HVA, DOPAC abd 5-HIAA at 1 and 2 hours post soman reflect increased formation due to the seizures state whereas increases in organic anions at 10 and 24 hours post soman seizures are likely due to impaired function of energy-dependent probenecid-sensititive anion transporters in the choriod plexus, possibly mediated by free radical damage to mitochondria. The fact that in general organic anions don't accumulate greater in the pirifrom cortex (area with extensive injury) compared to other areas shown (minimal injury) suggests that the inhibition of cellular pumps is not a major contributor to the observed increases in brain organic anions. This data was presented at the 1997 Neuroscience meeting (Pazdernik, T.L., R. Cross, S.R. Nelson and F.E. Samson. The nerve agent soman attenuates the clearance of metabolic anions out of the brain. Soc. Neurosci. 23: 749.13, 1997) will be sumbitted for publication.

The best indicator of tissue oxidative stress is a reduction in tissure glutathione (GSH). GSH was reduced 50 % in piriform cortex and 25 % in hippocampus 24 hours after soman exposure (Figure 39). GSH in cerebellum was unchanged after soman (control = 1.90 ± 0.21; 1 hour = 2.01 ± 0.25; 24 hour = 1.86 ± 0.36 μmoles/ gm tissue). GSSG (glutathione dimer) and PSH (protein thiols) were variable. The changes in glutathione composition in piriform cortex and hippocampus are indicative of an oxidative stress in these brain regions following nerve gas induced seizures.

From the extensive work that we have done over the past few years on redox active substances in biological fluids and tissues, we have concluded that iron complexes such as

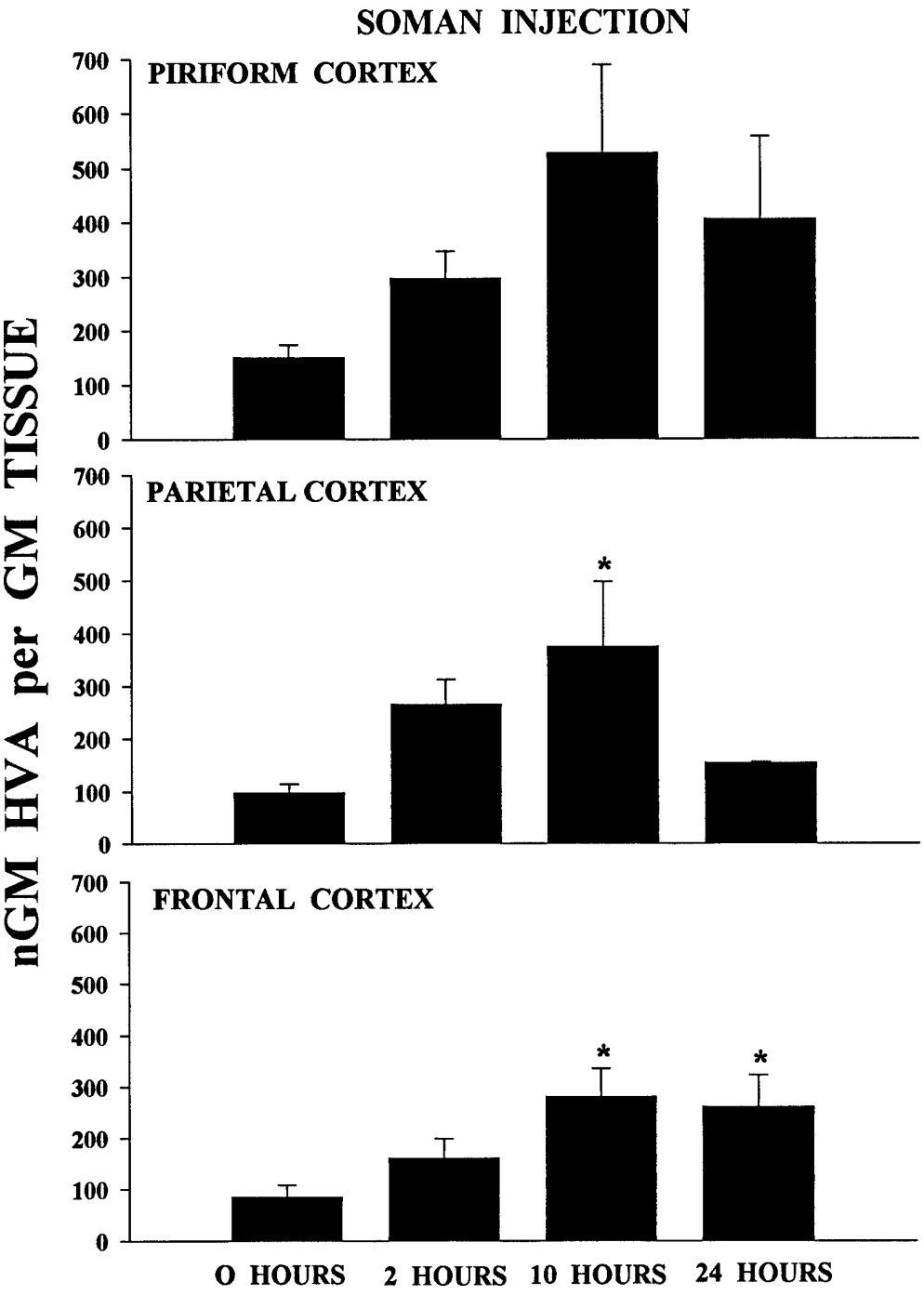

Figure 35. Concentrations of HVA (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after an injection of soman (80-90 μg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses (N=2 to 10). * = significantly different from 0 HOURS at $P<0.05$ using one-way ANOVA and Dunnett's post-hoc test.

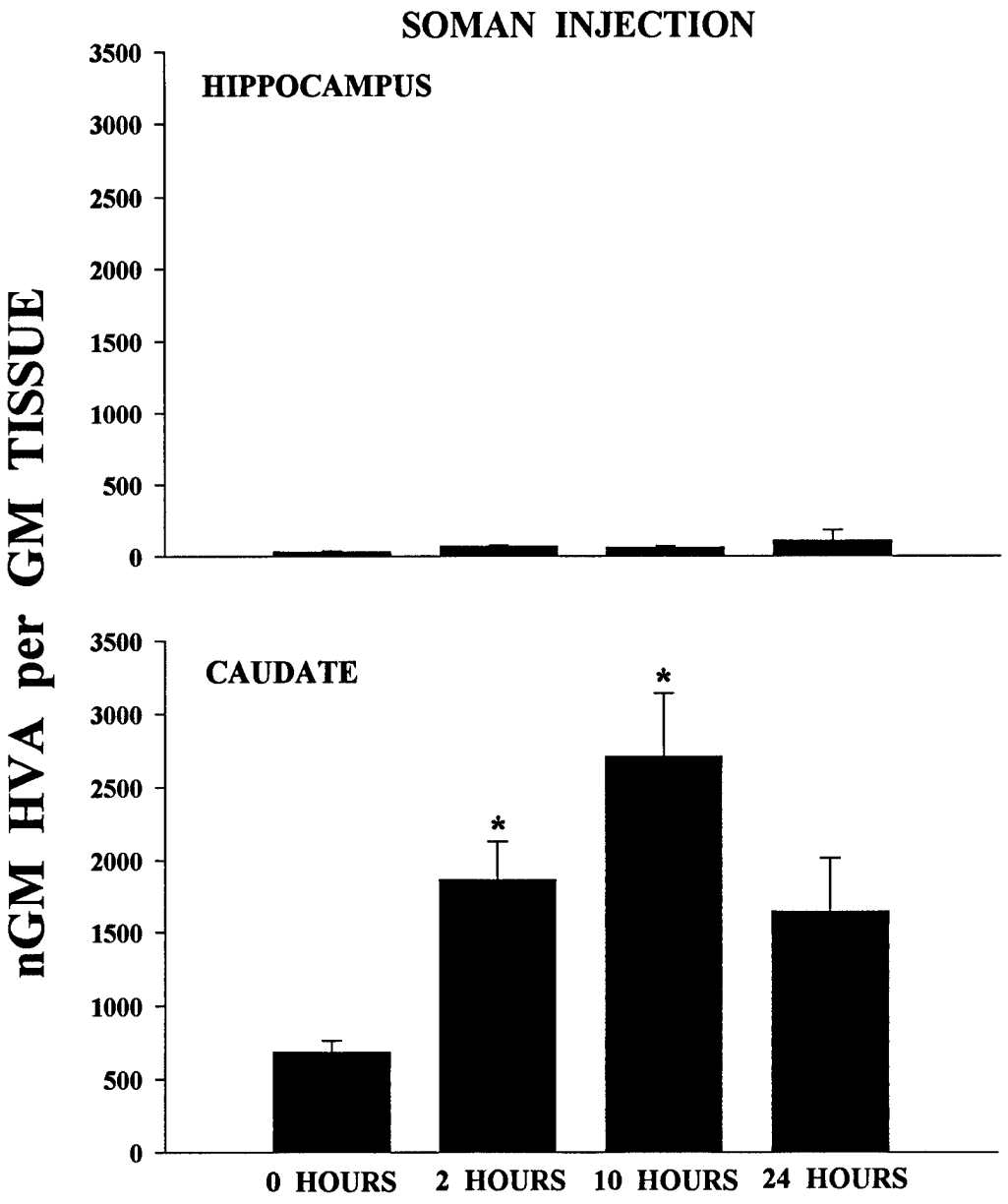

Figure 36. Concentrations of HVA (ng/g tissue) in tissue homogenates of hippocampus and caudate after an injection of soman (80-90 mg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses. (N= 2 to 9) * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

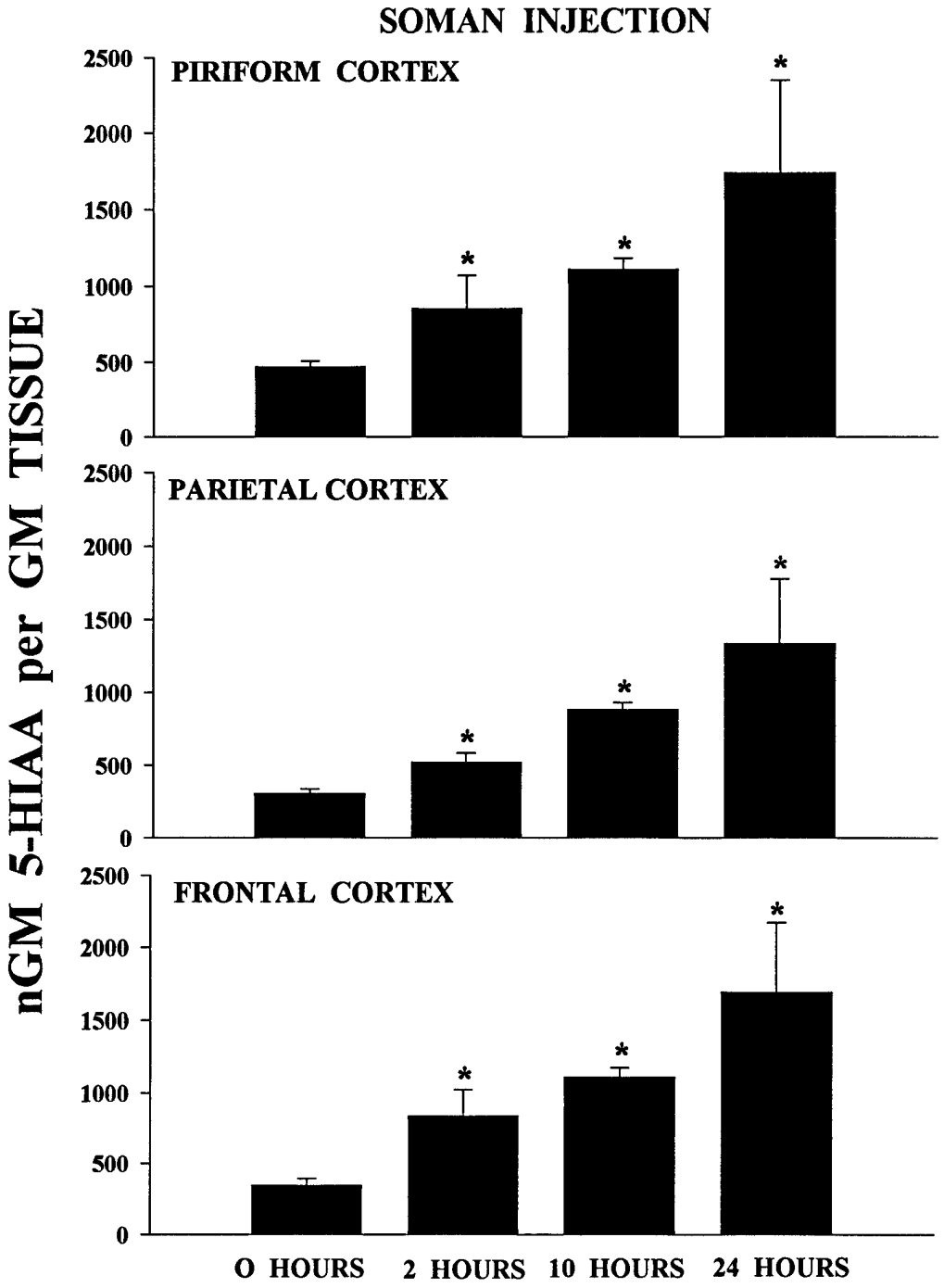

Figure 37. Concentrations of 5-HIAA (ng/g tissue) in tissue homogenates of piriform, parietal and frontal cortices after an injection of soman (80-90 μg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses (N=2 to 10). * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

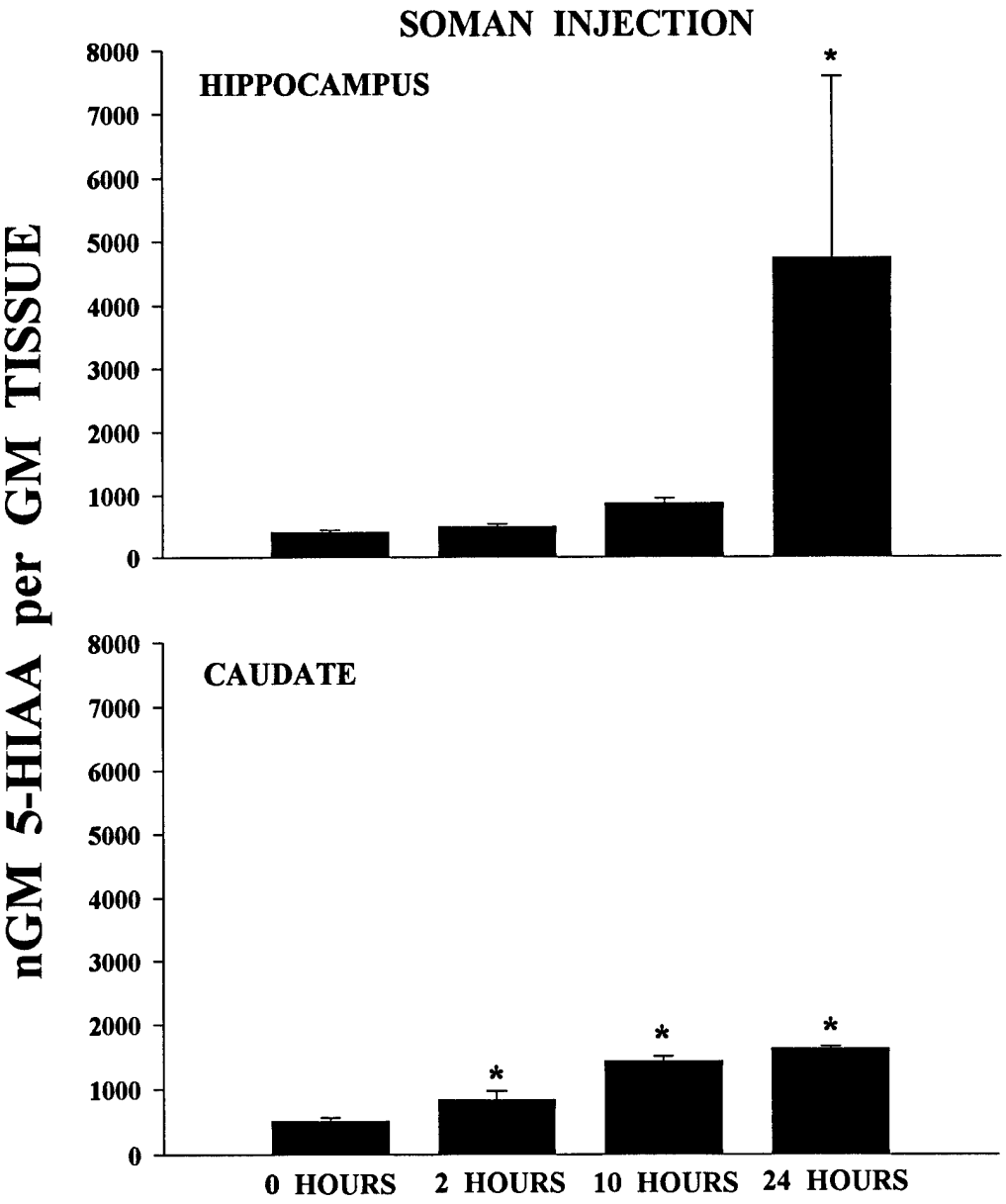

Figure 38. Concentrations of 5-HIAA (ng/g tissue) in tissue homogenates of hippocampus and caudate after an injection of soman (80-90 mg/kg; im). All rats developed typical soman-induced seizures. Salicylate (50 mg/kg; ip) was given 2.5 hours before sacrifice. Brains were dissected at times indicated and immediately homogenized as in methods for analyses. (N= 2 to 10) * = significantly different from 0 HOURS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

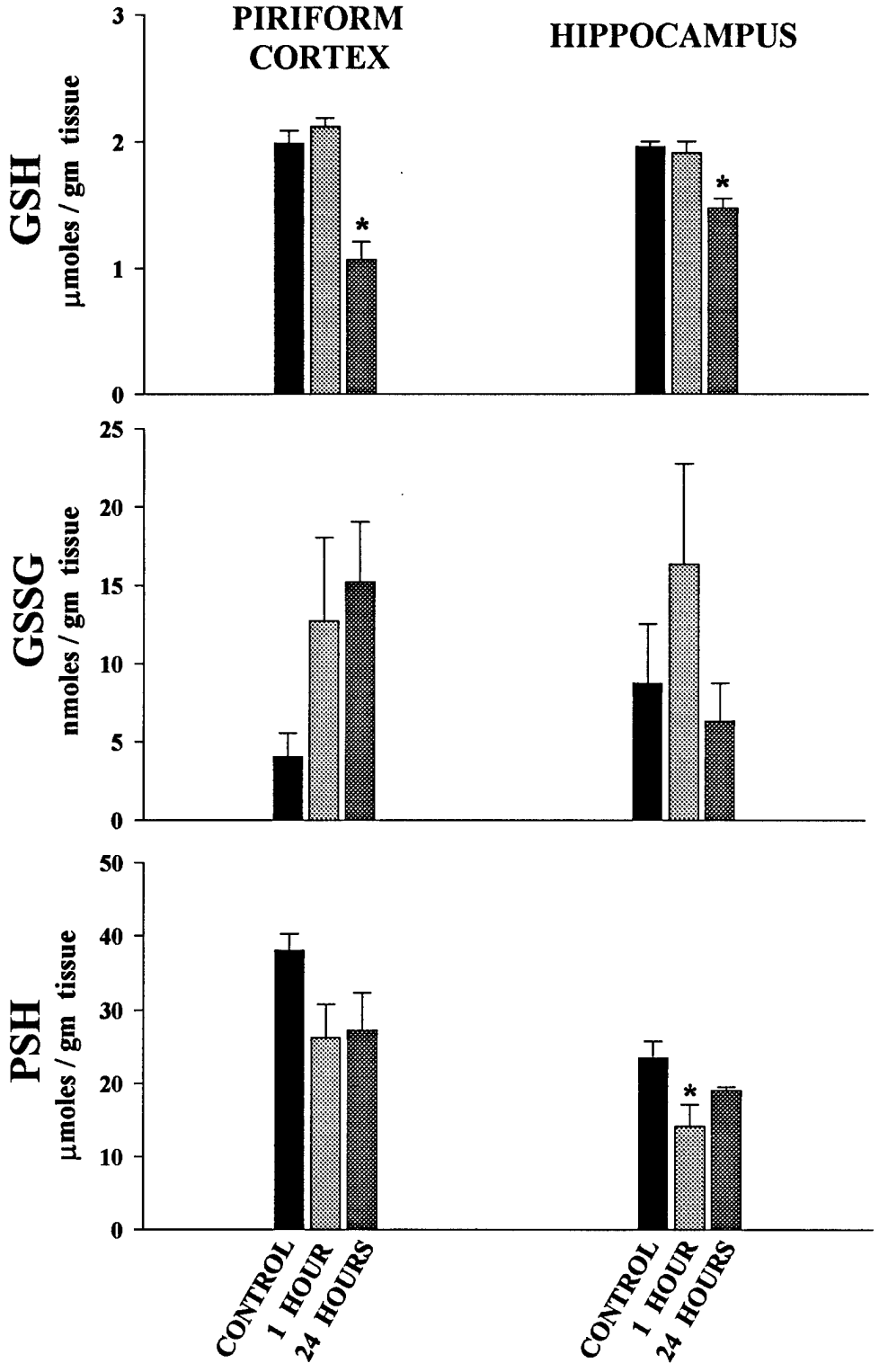

Figure 39. Concentrations of GSH (µmoles/g tissue), GSSG (nmoles/g tissue) and PSH (µmoles/g tissue) in piriform cortex and hippocampus after rats were injected with soman (85µg/kg; im). All rats had typical soman-induced seizures (N=5). * = significantly different from CONTROLS at P<0.05 using one-way ANOVA and Dunnett's post-hoc test.

ascorbate/iron/peroxide cause site specific damage. In order to determine if there is an increase in "catalytic" iron in the brain associated with soman-induced seizures, one of us (S.R. Nelson) developed a micro assay to measure "catalytic" iron. "Catalytic" iron (μmol/kg.d.wt.) was significantly higher 3 days after soman-induced seizures than in controls in brain regions with damage (ie., piriform cortex; 202 %, thalamus; 130 %, hippocampus; 125 %) but not in regions with minimal damage (ie., parietal cortex; 96 %, caudate; 96 %) (Figure 40). The increase in "catalytic" iron was not present at 1 or 24 hours after seizures. See Figure 41 for data on piriform cortex. The "catalytic" iron assay system employed in this study uses a 30 minute tissue extraction period with 0.5 mM EDTA. This extraction system removes loosely bound and heme iron from tissue but appears not to extract ferritin bound iron. Since significant increases in "catalytic" iron was only found at three days post seizure in areas where damage was detected, it is likely that the increase in "catalytic" iron is coming largely from heme iron associated with cell breakdown and micro hemorrhages from tissue breakdown. Marked changes in brain redox chemistry occurs with the onset of seizure activity. The continuation of these on-going redox changes finally over-whelms the oxidative defense mechanisms. This is seen in this study by the marked reduction of tissue GSH at 24 hours. Once the oxidative defense mechanisms are compromised, the oxidative response is fueled by iron released from hemoglobin, transferrin, and ferritin by either lowered tissue pH or oxygen radicals (Hall, 1993). This leads to radical-initiated peroxidation and cell lysis and tissue destruction. The accumulation of heme iron from cell breakdown and hemorrhages further fuels the oxidative damage. These reactions may play a role in the progressive spread of brain damage observed 30 days after soman. This data was presented at the 1998 Bioscience Review and will be presented at the 1998 Soc. of Neuroscience Meeting.

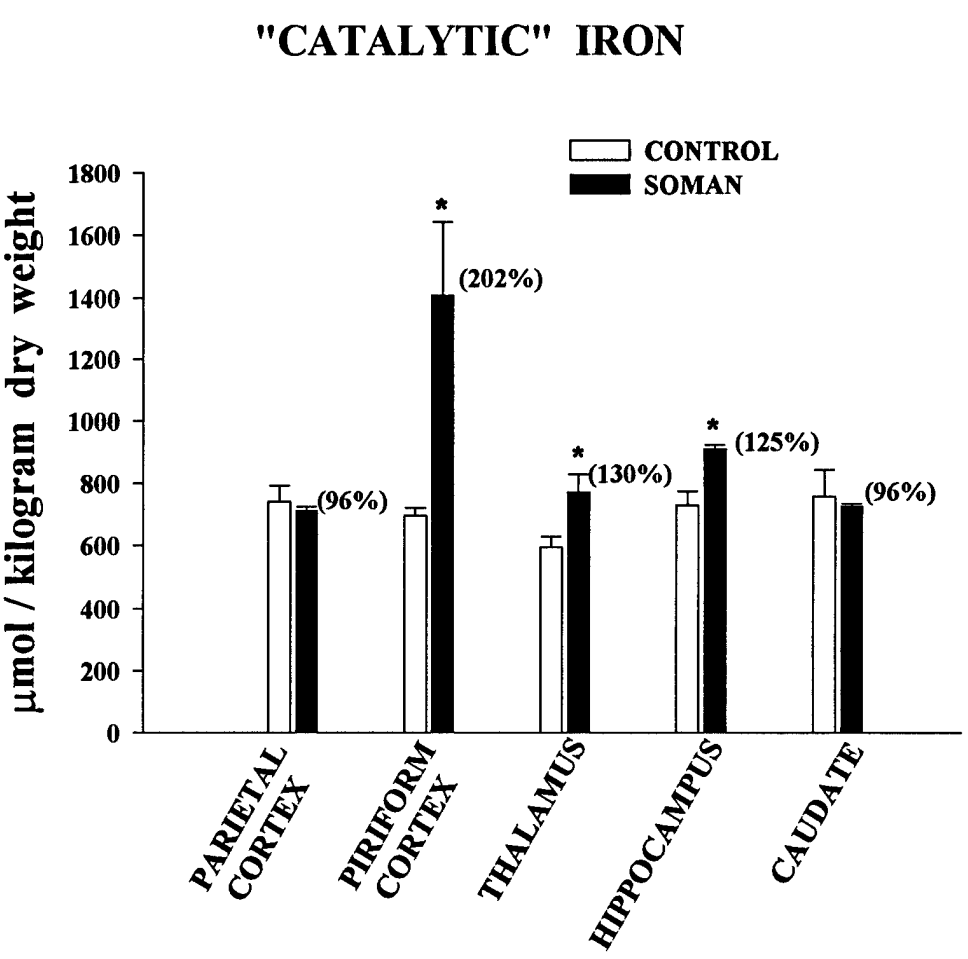

Figure 40. Regional levels of "catalytic" iron (µmole/kg dry weight) in tissues obtained from control rats and rats exposed to a seizurogenic dose of soman 72 hours prior to sacrifice (N=3). * = significantly different from CONTROL at P<0.05 using student t-test.

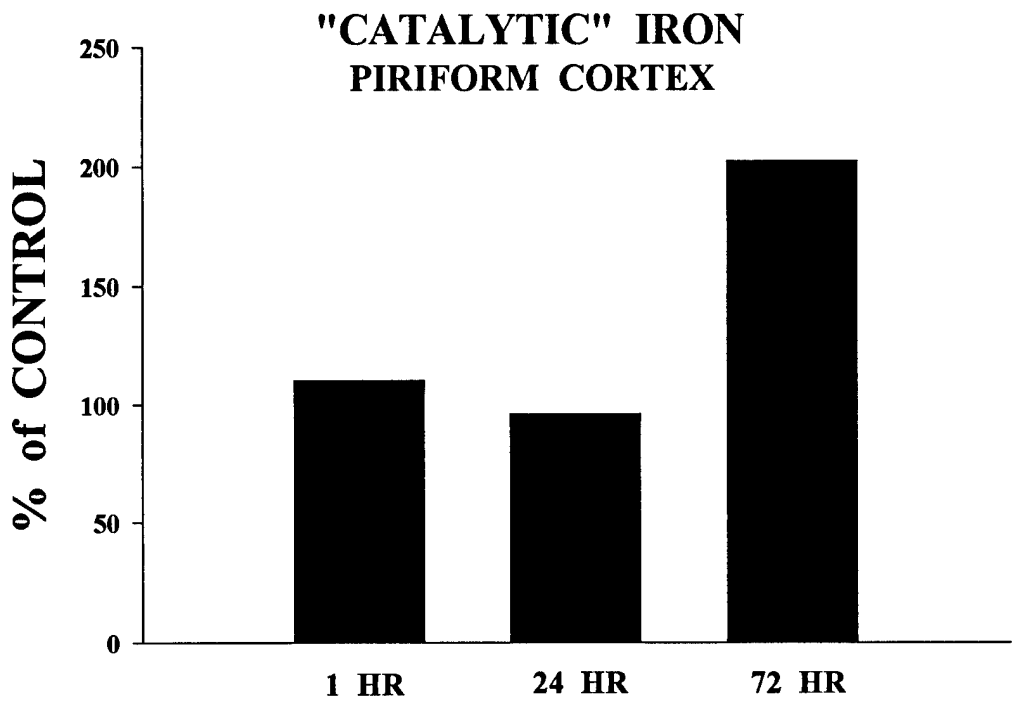

Figure 41. "Catalytic" iron expressed as percent of control in piriform cortices obtained from rats 1, 24 or 72 hours after a seizurogenic dose of soman.

Task 2. Gene expression studies: In preparation for this contract we initiated studies with Dr. Glen Andrews (Department of Biochemistry and Molecular Biology) to develop the capabilities to use gene expression studies as tissue biomarkers of free radical responses. The results of these studies are published in a paper entitled "Temporalspatial patterns of expression of metallothionein-I and -III and other stress related genes in rat brain after kainic acid-induced seizures", published in Neurochem. Int. 27:59-71 (1995).

From these studies, we conclude that seizure-induced neurodegeneration associated with the systemic administration of KA serves as a useful model to study the biochemical basis of brain injury. Systemic administration of KA was known to induce the expression of several genes, such as nerve growth factor (Gall et al., 1991), brain derived growth factors (Dugich-Djordjevic et al., 1992), cytokines (Yabuuchi et al., 1993), IEGs (Pennypacker et al., 1994) and heat shock proteins (Wang et al., 1993). In this study, we further demonstrate that there is a robust induction of MT-1 and HO-1 genes after KA-induced seizures, whereas MT-III, CuZn-SOD, GSTya and GPx genes are unaffected and only modest changes in Mn-SOD mRNA levels occur. The induction of MT-1 and HO-1 is seizure-dependent and is most prominent in areas where extensive damage occurs (i.e. piriform cortex). The concordant induction of MT-1 and HO-1 mRNAs indicate that oxidative stress is an important component in seizures and may be responsible for seizure associated brain damage.

Since metallothionein-1,2 and hemoxygenase-1 were most dramtically expressed during kainic acid seizures and since the co-expression of these two proteins are considered markers of oxidative stress, we focused on these two proteins after soman-induced seizures. Soman-induced

seizures caused a 14-fold increase in MT-1 mRNA in the piriform cortex by 4 hours post injection and levels remained elevated at 168 hours (7 days) (Figure 42). However, in the hippocampus, a biphasic induction of MT-1 mRNA levels occurred after soman, that is, a 5-fold increase at 2 hours, a return to basal levels, and then a secondary 4-fold induction at 24 hours (Figure 42). The levels of MT-1,2 and HO-1 in the piriform and parietal cortices and in the hippocampus 1 to 168 hours post soman injection are shown in Figures 43 and 44. MT-1,2 was increased 3-fold above control values in the parietal cortex by 24 hours post soman and remained elevated throughout the 7 day period. MT-1,2 increased more slowly in the piriform cortex but was 3.5-fold above control values at 3 days but returned to control values by 7 days. HO-1 was increased 1.5- to 2-fold above control values from 2 to 72 hours post soman. The increase of HO-1 above controls was greater at all time points in the piriform cortex (ie., 3.5-fold increase at 3 days) than in the parietal cortex. Neither MT-1,2 nor HO-1 changed dramatically in the hippocampus. Of these 3 tissues, the basal level of MT-1,2 was the lowest in piriform cortex and basal levels of HO-1 was the lowest in the hippocampus (Emerson et. al., in press).

Chemical-induced seizures are known to enhance the expression of several genes, such as nerve growth factors (Gall et. al., 1991; Dugich-Djordjevic et. al., 1992), cytokines (Yabuuchi et. al., 1993) immediate early genes (Le Gal LaSalle et. al., 1988; Shipley et. al., 1991; Pennypacker, et. al., 1994) and heat shock proteins (Wang et. al., 1993). We (Dalton et. al., 1995) found that kainic acid caused a very robust induction of two stress-related proteins (MT-1 and HO-1). This induction was seizure-dependent and most prominent in areas with extensive damage (ie., piriform cortex). The concordant induction of MT-1 and HO-1 is often cited as evidence of oxidative stress. Therefore, we focused on these two stress-related proteins during

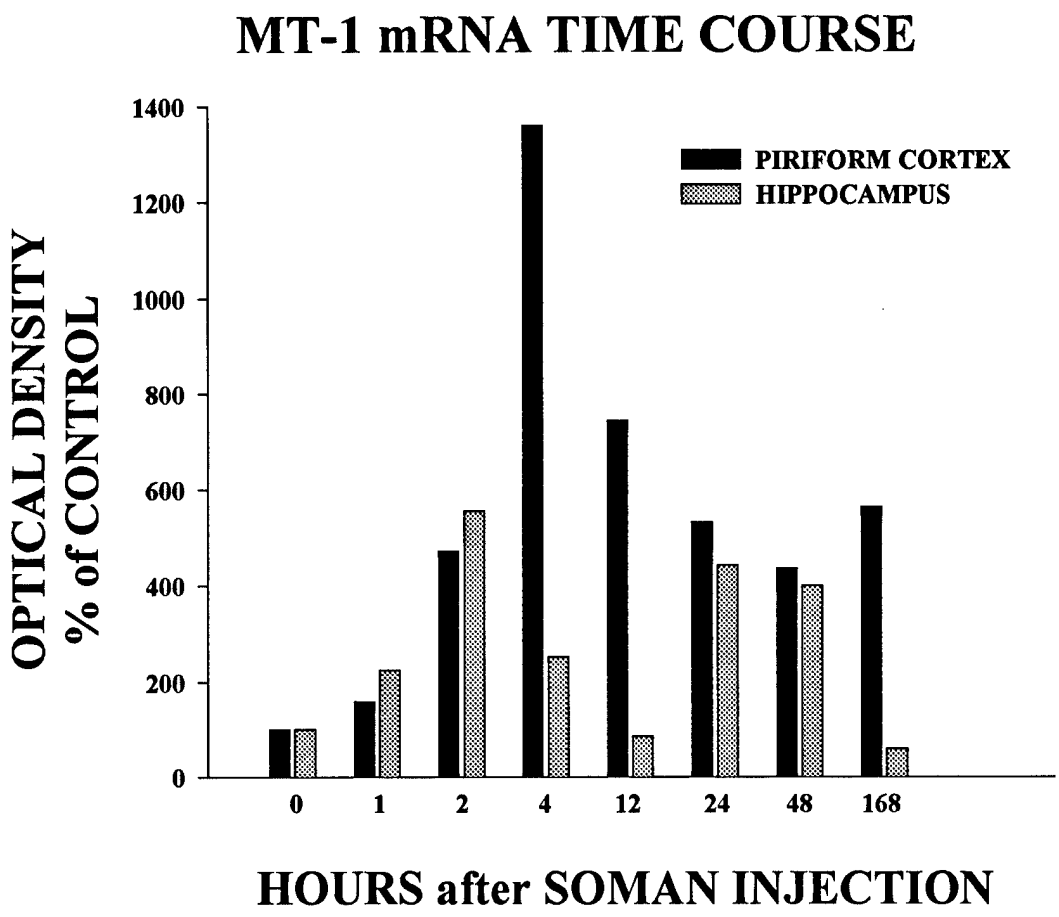

Figure 42. Quantitation of Northern blot analyses (percent of control) assessing mRNA levels for MT-1 in piriform cortex and hippocampus at various times after soman exposure (85-90 μg/kg; im). Pooled tissues from 3-4 rats were used.

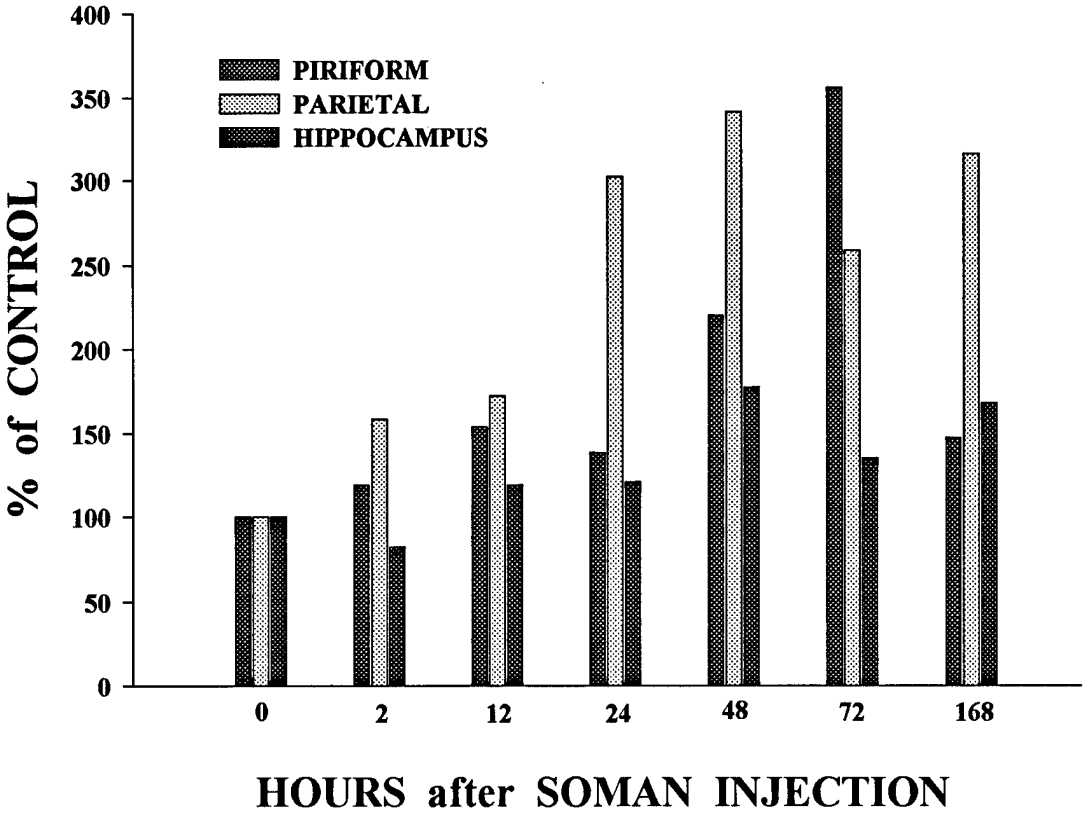

Figure 43. Quantitation of Western blot analyses (percent of control) assessing metallothionein-1,2 levels in piriform cortex, parietal cortex and hippocampus at various times after soman exposure (85-90 μg/kg; im). Shown are averages of 3 determinations using pooled tissue from 5 rats.

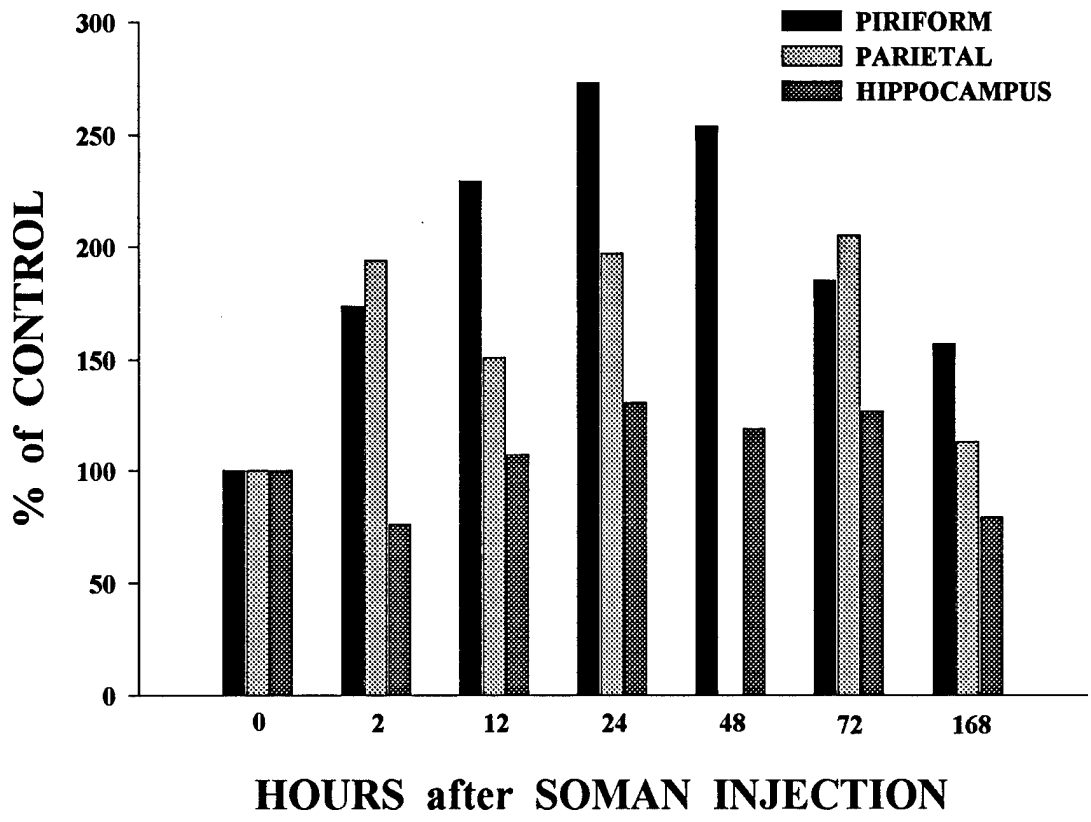

Figure 44. Quantitiation of Western blot analyses (percent of control) assessing heme oxygenase-1 levels in piriform cortex, parietal cortex and hippocampus at various times after soman exposure (85-90 μg/kg; im). Shown are averages of 3 determinations using pooled tissue from 5 rats. 48 hour time point is missing for parietal cortex.

soman-induced seizures. Soman-induced seizures also caused a similar time profile as kainic acid in the increase in MT-1 mRNA in the piriform cortex. On the other hand, the time course for changes in MT-1 mRNA in the hippocampus is biphasic just as is the extracellular glutamate time course response in the CA1 region following soman (Lallement et. al., 1991). Zinc is colocalized and coreleased with glutamate and attenuates the actions of glutamate at the NMDA receptor. The highest levels of vesicular zinc occur in limbic associated areas (Frederickson, 1989) and interestingly MT-3 mRNA is also highest in these areas (Aschner, 1996), suggesting a possible functional role of MT's in neuronal zinc disposition and release. Thus, a robust increase in MT-1 mRNA may indicate an excessive release of glutamate and zinc. Recent in vivo and in vitro studies with Hmox1-deficient mice indicate that HO-1 is crucial for iron homeostasis (Poss and Tonegawa, 1997a, 1997b). The enhanced expression of stress-related proteins has been viewed as an adaptive response to seizures (Massa et. al., 1996). Therefore, it may seem paradoxical that, if these proteins serve a protective role, the induction of mRNAs is the greatest in areas where the damage is the greatest. However, the increase in protein levels is modest in comparison to the increase in message. Baille-Le Crom et al.,(1996) found that soman induces transcription of c-fos and HSP70 mRNAs in piriform cortex and hippocampus but that there was a lack of HSP70 gene translation in the extensively damaged piriform cortex. Thus, if "stress" proteins are protective, their induction is too little and too late. However, an increase in "stress" proteins does indicate brain regions are "stressed" and may give insight into the type of stress involved. For example, an increase in MT-1 may reflect an increase in zinc/glutamate release whereas an increase in HO-1 may indicate an increase in delocalized iron and an on-going oxidative stress.

Task 3. Pharmacological Interventions: This phase of the study was not successful. The rats that we are using at the present time have an extremely sharp dose response curve to soman and, thus, it is difficult to get rats to survive seizurogenic doses of soman. We need to change our experimental paradigm more along the approaches that are now being employed by scientists at Aberdeen Proving Ground. They almost exclusivley use peripheral protection agents in order to give rats a large enough dose of soman to causes intense seizures but yet allow the rats to survive in order to assess brain damage and to evaluate neuroprotective efficacy of test componds. Our data strongly implicates that redox changes do occur after cyanide and soman. Therefore, pharmacological agents that interfer with these processes should provide protection especially against delayed brain injury.

CONCLUSIONS

We studied how soman, kainic acid or cyanide change the redox status of the piriform cortex. This was done by sampling extracellular fluid by intracerebral microdialysis and observing changes in regional brain tissues for redox active molecules and the gene expression of protein biomarkers of oxidative stress. Soman and kainic acid were administered to rats at doses that induce seizures and seizure-induced brain damage. Cyanide is given by intermittent intravenous infusion to keep the rats comatose. Under conditions resulting in severe pathology, there was an increase in piriform cortex extracellular glutamate and taurine (a biochemical marker of cellular swelling) as expected with the excitotoxic amino acid cascade of brain cell injury. All three neurotoxins cause an increase in extracellular ascorbate and urate indicating an oxidative stress. Our extensive test tube experiments with salicylate hydroxylation as a reporter for hydroxyl radical indicate that delocalized metals in brain extracellular fluid (e.g. human CSF) can generate hydroxyl radicals but that these radicals are likely rapidly inactivated by the extracellular milieu. Our data with the use of salicylate as an in vivo reporter to trap hydroxyl radicals during and after soman-induced seizures in rats are inconclusive. This is likely because in vivo ascorbate/iron/peroxide complexes cause site specific oxidative damage. Indeed, we have an increase in "catalytic" iron and a decrease in tissue glutathione after soman. Kainic acid exposure increased nitric oxide levels in hippocampal microdialysis perfusates in both anesthetized and awake rats. Also, both soman and cyanide cause significant increases in 5-hydroxyindole acetic acid (HIAA), homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC), indicating either an extensive release of serotonin and dopamine and/or a failure to transport these acid metabolites from extracellular fluid. Kainic acid induced-seizures are

associated with marked increases in mRNAs for metallothionein-1 and heme oxygenase-1 and lesser but significant increases for c-fos, heat shock protein-70 and interleukin-1ß. The induction of these mRNA's are seizure dependent, and are greater in brain areas with extensive damage than in areas with minimal damage (e.g., frontal cortex or cerebellum). The prolonged and robust concordant expression of metallothionein-1 and heme oxygenase-1 may reflect the oxidative stress produced by kainic acid-induced seizures. In addition, the induction of interleukin-1ß gene expression suggests an inflammatory response in brain regions damaged by kainic acid-induced seizures. Very similar changes in metallothionein-1 and heme oxygenase-1 were observed after soman-induced seizures. We postulate that less reactive ascorbate/metal/peroxide complexes are critical oxidizing intermediates in brain injury. Characterizing the redox changes associated with oxidative and inflammatory responses contributes to a better understanding of neurotoxin related brain injury. Clearly, the redox state is important in neurotoxin-induced brain injury. Knowing this redox chemistry will facilitate the development of pharmacological strategies to intervene with a free radical, final common pathway.

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